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BOMBESIN AND CHOLECYSTOKININ REGULATE FEEDING BEHAVIOR AND ANTERIOR PITUITARY HORMONE RELEASE IN GOLDFISH

by

BEVERLY A. HIMICK

A thesis submitted to
the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of **Doctor of Philosophy**.

DEPARTMENT OF ZOOLOGY
EDMONTON, ALBERTA
FALL, 1994

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I hereby permit Ms. Beverly Ann Himick to include research data, in which we did in collaboration, in her doctoral thesis from the following papers:

Himick, B.A. and Peter, R.E. CCK/gastrin-like immunoreactivity in the brain and gut and CCK suppression of feeding behavior in goldfish (in press) Amer. J. Physiol.: Reg. Integr. Comp. Physiol.) (1994).

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Date: 6 June 1994

This thesis is dedicated to my mother and the loving memory of my father

your constant support during my studies will always be remembered.

Abstract

Cholecystokinin (CCK)/gastrin-like and bombesin (BBS)/gastrin releasing-like peptides (GRP) have received little attention in fish, but play pivotal roles in the regulation of feeding behavior, pituitary hormone secretion, gut motility, and gallbladder contraction in mammals. In the present study, the distribution of CCK/gastrin-like and BBS/GRP-like immunoreactive (IR) peptides, and specific CCK/gastrin and BBS/GRP binding sites were examined in the central nervous system of the goldfish, *Carassius auratus*. Additionally, the *in vivo* and *in vitro* effects of CCK and BBS on feeding behavior and anterior pituitary hormone secretion in goldfish were investigated.

CCK/gastrin-like and BBS/GRP-like IR material, and specific, high affinity ^{125}I-BH-CCK8-s (sulfated CCK8) and ^{125}I-[Tyr-4]-BBS-14 binding sites were distributed throughout the goldfish forebrain, midbrain and hindbrain. The presence of IR material and binding sites within the ventro-posterior hypothalamus (brain feeding center), suggests that CCK- and BBS-like peptides may be involved in the central control of food intake in fish. When injected into the third brain ventricle of goldfish, both BBS and CCK8-s were highly effective in suppressing feeding. Likewise, when administered intraperitoneally (ip), BBS and CCK8-s caused a dose-dependent suppression in food intake. Nonsulfated CCK8 was not effective in suppressing feeding following ip injection. In the pituitary, CCK/gastrin-like IR was co-distributed among the somatotropes and gonadotropes of the proximal pars distalis, while BBS/GRP-like IR fibres were prevalent within the neurointermediate lobe; occasionally cells containing BBS/GRP-like IR material were localized in the pars distalis. When goldfish pituitary fragments were challenged with pulses of CCK8-s or BBS *in vitro*, an increase in both growth hormone (GH) and gonadotropin (GtH-II) secretion resulted.

These studies provide evidence that neuropeptides are involved in the regulation of food intake in goldfish, and that BBS and CCK binding sites are also present in the brain feeding center. BBS and CCK may also play a role in modifying the release of GH and GtH-II from the anterior pituitary of goldfish.

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Chapter 1

Literature Review

1.1 General Introduction

Peptides in the bombesin (BBS)/gastrin-releasing peptide (GRP) family and cholecystokinin (CCK)/gastrin family have received little attention in fish, but play pivotal roles in the regulation of feeding behavior, pituitary hormone secretion, gut motility, and gall bladder contraction in mammals. The focus of my doctoral thesis has been on investigating the neuroendocrine and feeding actions mediated by the two neuropeptides, BBS and CCK, in the goldfish, *Carassius auratus*. To provide evidence that BBS- and CCK-like peptides exist within the goldfish, the immunohistochemical distributions of both peptides were mapped within the brain and pituitary, as well as within areas of the gastrointestinal tract. Finally, to provide information on the sites of action of BBS and CCK-like peptides within the goldfish central nervous system, specific, high affinity binding sites for BBS and CCK were localized and characterized within the goldfish brain and pituitary. These studies are unique in that they are the first to document in detail that neuropeptides are involved in the regulation of feeding behavior in lower vertebrates, and that their high affinity receptor binding sites are localized in the brain feeding center. The present studies also suggest that a relationship may exist between altered feeding behavior and anterior pituitary hormone secretion in fish.

1.2 Feeding Behavior in Fish

1.2.1 Fish Regulate the Amount of Food they Consume

Studies indicate that fish regulate the amount of food that they consume, although limited information exists regarding the physiological mechanism(s) and neuronal circuitry involved in such control. Early experiments by Rozin and Mayer (1961; 1964) reported that goldfish will distribute their feeding responses fairly evenly over the light phase and that the amount of food consumed is dependent on both ambient temperature and the nutrient content of the food. For example, if goldfish are presented with small pellets, they consume more food relative to the intake of larger food pellets in order to

satisfy their appetite. If the same fish are exposed to a $10^{o}C$ drop in ambient temperature, they will decrease their food intake by one half to one third (Rozin and Mayer, 1961).

Other fish species which have been reported to regulate the amount of food they consume include the yearling sockeye salmon *Oncorhynchus nerka* (Brett, 1971; Bilton and Robins, 1973), the winter flounder *Pseudopleuronectes americanus* (Tyler and Dunn, 1976), the rainbow trout *Oncorhynchus mykiss* (Lee and Putnam, 1973), and the northern pike *Esox lucius* (Johnson, 1966). If flounder are starved or deprived of food, they feed more intensely to compensate for loss of body reserves (Tyler and Dunn, 1976). Grove *et al.* (1978) demonstrated that groups of *O. mykiss* will consume a greater amount of trout pellets if the food is diluted with kaolin, while Grayton and Beamish (1977) reported that food intake in trout remains constant despite changes in the frequency of feeding. Peter (1979) suggested that like mammals, fish adjust their body size to a "set point" value depending on their energy stores, current size, and season. This is subsequently reflected in the amount of food the fish eats.

1.2.2 The Brain and Fish Feeding Behavior

How do fish regulate the amount of food they consume? It is likely that as in higher vertebrates, the teleost brain plays a pivotal role in controlling feeding activities and satiation, or the termination of food intake. Based on earlier anatomical studies illustrating gustatory and medial forebrain bundle connections to the inferior lobe of the hypothalamus (Herrick, 1905; Crosby and Showers, 1969), as well as electrical stimulation studies on specific brain areas in the carp *Cyprinus carpio* (Redgate, 1974), bluegill sunfish *Lepomis macrochirus*, and in the tilapia *Tilapia macrocephala* (Demski and Knigge, 1971; Demski, 1973; for review Demski, 1983), areas within the fish ventro-posterior hypothalamus and the hypothalamic inferior lobes appear to be involved in the regulation of feeding activity (Fig. 1.1, after Demski 1983). For example, Savage and Roberts (1975) reported that in the goldfish, low threshold electrical stimulation in areas just dorsal to the lateral recess of the third venticle and slightly below the nucleus preglomerulosus of the hypothalamus caused an increase in feeding activity. On the contrary, bilateral lesions within the hypothalamic lateral or inferior lobes of goldfish

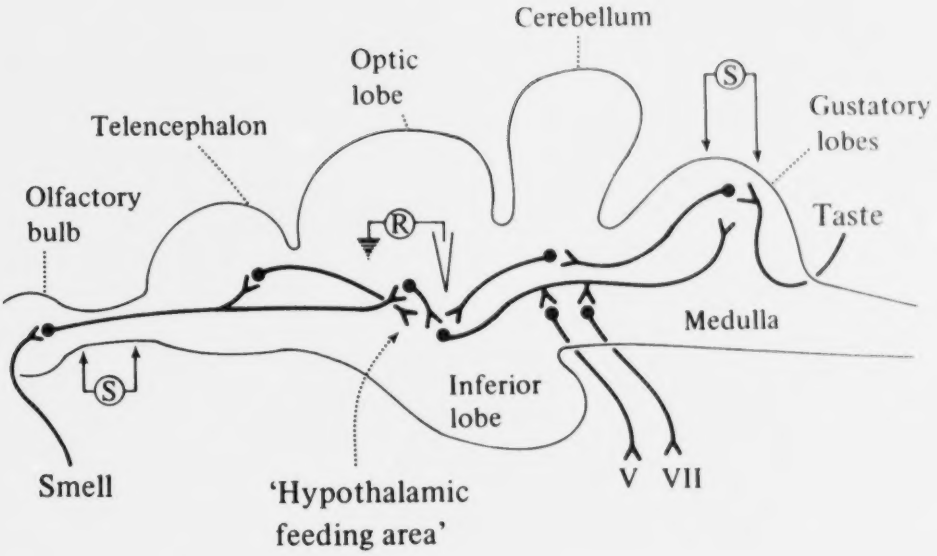

Fig. 1-1. Sagittal section of the goldfish brain illustrating the hypothalamic feeding area. Based on electrical stimulation studies, the hypothalamic feeding center in fish has been identified within the ventro-posterior hypothalamus and hypothalamic inferior lobes. Connections to the hypothalamic feeding area from other brain areas are also illustrated. V = motor root of the trigeminal nerve; VII motor root of the facial nerve. (after Demski, 1981).

produced a dramatic reduction in feeding behavior. In line with this, Redgate (1974) reported that in *C. carpio*, stimulation of the hypothalamic inferior lobe resulted in feeding responses. Electrical stimulation of nerve fibres which enter the ventro-posterior hypothalamus also produce alterations in feeding behavior. This includes second- and third- order gustatory fibres which terminate in the subrotundal area, olfactory bulb projections which end just medial to the subrotundus region, and innervation by medullary gustatory lobe fibres (Finger, 1978; for review Demski, 1981). In goldfish, the feeding response induced by a food odour can be abolished if the lateral part of the olfactory tract which enters into the lateral hypothalamus is sectioned (Stacey and Kyle, 1983). Areas within the medial telencephalon have also been shown to partially alter feeding activity when stimulated (Savage, 1971). These studies indicate that the inferior lobe of the teleost hypothalamus is an integrating center for neuronal stimuli involved in the regulation of feeding behavior. It remains unknown, however, which specific neuropeptides and neurotransmitters play a role in the teleost hypothalamic feeding area to regulate food consumption.

1.3 Pituitary Growth Hormone and Feeding

1.3.1 Mammals

In mammals evidence suggests that feeding alters circulating growth hormone (GH), and that elevated serum GH may actually facilitate food intake. Rats which are chronically implanted with intra-cardiac catheters exhibit enhanced food intake during periods that coincide with the release of GH into the blood, while feeding activity is reportedly reduced during the GH peak-to-peak periods in which bursts of insulin secretion occur (Even *et al.*, 1978). Moseley *et al.* (1988) also described a significant increase in serum GH in steers just prior to feeding, followed by a decline of basal GH levels for 2 to 3 hours after feeding.

The established hypothalamic regulators of pituitary GH, somatostatin (SRIF) and GH releasing factor (GRF), have also been implicated in the regulation of mammalian feeding behavior. Lin *et al.* (1987) reported that infusions of SRIF into the lateral hypothalamus of rats resulted in a reduction in food intake. In line with this, Ho *et al.* (1989) demonstrated that hypothalamic injections of SRIF into rats caused a decrease in

food intake, whereas lowering endogenous hypothalamic SRIF levels by cysteamine administration elevated feeding. Feifel and Vaccarino (1990) observed that while low doses of SRIF injected into the lateral ventricles of rats caused an increase in feeding, higher doses of injected SRIF produced a decrease in food intake, indicating a biphasic action of SRIF on food consumption. GRF also appears to stimulate feeding behavior when administered centrally. Dickson and Vaccarino (1990) reported that injection of GRF into the suprachiasmatic nucleus-medial preoptic area resulted in a dose dependent elevation in food intake, increasing both meal length and rate of eating, while Vaccarino *et al.* (1991) demonstrated that injection of GRF antiserum into this same brain area significantly attenuated the normal feeding behavior.

1.3.2 Fish

In fish, studies suggest that pituitary GH may be important in the regulation of feeding activity and fish growth (for review Donaldson *et al.* 1979; Weatherley and Gill, 1987), through both an increase in food consumption and by an improved food conversion efficiency (Higgs *et al.* 1975; Markert *et al.* 1977; Kayes 1978). For example, Higgs *et al.* (1975) reported that in yearling coho salmon, intraperitoneal (ip) injections of bovine GH resulted in an increase in mean wet body weight, while in the hypohysectomized black bullhead *Ictalurus melas*, administration of bovine GH produced an increase in food conversion efficiency (Kayes, 1978). Furthermore, in hypophysectomized *I. melas*, Kayes (1978) reported reduced food-seeking behavior and decreased rate of feeding. This effect was not observed however in hypophysectomized fish treated with bovine GH. Injections of bovine GH into *O. kisutch* also increases voluntary food intake and food conversion (Markert *et al.*, 1977). It is suggested that GH may alter appetite by either directly affecting control centres in the hypothalamus or by inducing a number of metabolic changes that feed back on the control centers in the hypothalamus.

1.4 The Role of Neuropeptides in the Regulation of Feeding Behavior

The regulation of feeding behavior is a complex process involving neuropeptide/ neurotransmitter signals from both the gastrointestinal tract and within the central nervous system. Peripherally, gastrointestinal peptides are released from the stomach and intestine in response to a meal where they act to regulate the mechanical events associated with digestion and subsequently also act to terminate food intake. Concomitant with this event is the modified release of several neuropeptides/neurotransmitters within areas of the hypothalamus which alter the tonicity of the feeding drive system (Morley, 1987; McCoy and Avery, 1990; Silver and Morley, 1991).

In mammals, several gastrointestinal peptides which function in the peripheral satiety system have recently been reported to exist within specific brain areas where they act as neuropeptides to regulate feeding behavior. Of these, BBS and sulfated CCK8 (CCK8-s) have received considerable attention, since both have been shown to acutely suppress food intake following binding to their specific, high affinity receptors at the level of the gut or within the hypothalamus (Baile *et al.*, 1986; Morley, 1987; McCoy and Avery, 1990; Silver and Morley, 1991). When administered into the brain, both CCK and BBS act directly or interact with other brain neurotransmitters/neuropeptides, such as neuropeptide Y, which causes a change in the tonicity of the feeding drive system in the lateral hypothalamus (for reviews Morley, 1987; McCoy and Avery, 1990; Silver and Morley, 1991). When administered peripherally, BBS and CCK have been reported to alter such gastrointestinal events as exocrine and endocrine pancreatic secretion, gastrointestinal motility, gastrin and gastric acid secretion, and gallbladder contraction (for reviews Morley, 1987; McCoy and Avery, 1990; Silver and Morley; 1991), all of which contribute to the initiation of satiety.

While the actions of BBS and CCK are established in mammals, the possible roles that these peptides may play in the regulation of feeding behavior in lower vertebrates remains unknown. The growth and perpetuation of a species requires that behaviors associated with the acquirement of food sources (searching and locomotor responses, food intake, ingestion) be precisely regulated on both a daily basis and during the season when growing and reproductive phases are shifting. While the long-term effects of

hormonal influence on feed efficiency and growth have been well documented in fish, there exist no studies which have examined the acute regulation of feeding behavior in fish. Such a fundamental unanswered question formed the basis of this thesis, where the actions of two neuropeptides, BBS and CCK, were investigated in the goldfish. This involved examining the localization and distribution of BBS- and CCK-like immunoreactive (IR) peptides in the brain and pituitary, their respective receptors within the central nervous system, and the effects that these two peptides exert on feeding behavior and anterior pituitary hormone release in the goldfish. These studies are unique in that they provide initial evidence that neuropeptides participate in the regulation of feeding behavior in fish and that pituitary GH may interact in this event.

1.5 Bombesin

1.5.1 Characterization and Distribution

The tetradecapeptide bombesin (BBS) was first isolated from skin extracts of the fire bellied toad, *Bombina bombina* (Anastasi *et al.*, 1971), but has since been shown to belong to a family of BBS-like peptides which are present throughout all vertebrate classes (Dockray *et al.*, 1979; Panula 1986; Costello *et al.*, 1991). BBS-like peptides have been divided into three subfamilies based on their penultimate and adjacent amino acids (Table 1.1); the BBS subfamily contains Leu as its penultimate amino acid, the ranatensin subfamily contains Phe as its penultimate amino acid, and the phyllolitorins occur in both a Leu (BBS-like) and Phe (ranatensin-like) form, but substitute a serine adjacent to the penultimate residue (for review Spindel *et al.*, 1993). In mammals, both BBS and gastrin releasing peptide (GRP), a 27 amino acid peptide which shares a similar decapeptide C-terminal sequence as BBS, are widely distributed within nerves of the central nervous system and the gastrointestinal tract, where they have been implicated in regulating feeding-related activities (for review McCoy and Avery, 1990). In the frog, BBS- and GRP-like peptides appear to be derived through two independent genes. While mRNA for GRP was shown to exist in only the brain and stomach of *Bombina orientalis*, BBS mRNA was localized in the brain, stomach and skin of this species (Nagalla *et al.*, 199).

Table 1.1. The amino acid sequence of the carboxy termini of several BBS -related peptides.

GRP 10 (Neuromedin C)	Gly-Asn-His-Trp-Ala-Val-Gly-His-**Leu**-Met-NH_2
Bombesin	Pyr-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-**Leu**-Met-NH_2
Trout GRP (*Oncorhynchus mykiss*)	Ser-Glu-Asn-Thr-Gly-Ala-Ile-Gly-Lys-Val-Phe-Pro-Arg-Gly-Asn-His-Trp-Ala-Val-Gly-His-**Leu**-Met-NH_2
Alytesin	Pyr-Gly-Arg-Leu-Gly-Thr-Gln-Trp-Ala-Val-Gly-His-**Leu**-Met-NH_2
Neuromedin B	Gly-Asn-Leu-Trp-Ala-Thr-Gly-His-**Phe**-Met-NH_2
Ranatensin	Pyr-Val-Pro-Gln-Trp-Ala-Val-Gly-His-**Phe**-Met-NH_2
Litorin	Pyr-Gln-Trp-Ala-Val-Gly-His-**Phe**-Met-NH_2

1.5.1.1 Fish

In fish, BBS/GRP-like IR peptides have been detected within neurons and endocrine cells of the gastrointestinal tract (for review Bjenning and Holmgren, 1988; Holmgren and Jonsson, 1988; Bjenning *et al.*, 1990), as well as in the cardiovascular system (Bjenning *et al.*, 1990). For example, in the Atlantic cod (*Gadus morhua*) BBS-like IR material was reported within muscle layers of the cardiac stomach and outer curvature of the pyloric stomach (Holmgren and Jonsson, 1988). BBS-like IR has also been described in nerve fibres of the anterior mesenteric and coeliac arteries, and within intrinsic vessels of the gut in *Squalus acanthias* (Bjenning *et al.*, 1990). In this species, perifusion of BBS has been shown to alter blood flow of isolated, stomach tissues (Bjenning *et al.*, 1990). Using gel chromatography, the presence of several BBS/GRP-like peptides in the fish gut have been shown. In intestinal extracts of the elasmobranch *Scyliorhinus canicula,* a 25-amino acid GRP-like peptide and a shorter peptide, similar in structure to neuromedin C, have been isolated (Conlon *et al.*, 1987). In another elasmobranch, *Scyliorhinus stellaris*, two forms of BBS/GRP-like peptides were also reported, with one peptide exhibiting a similar elution profile as that for amphibian BBS (Cimini *et al.*, 1985). Several BBS/GRP-like IR peptides have also been described from extracts of the spiny dogfish gastrointestinal vessels and gut muscle and mucosal layers; one fraction of IR material shared chromatographic properties with BBS. More recently, a GRP-like peptide has also been isolated and sequenced from the stomach of the rainbow trout, *O. mykiss* (Jensen and Conlon, 1992).

Only one study to date has documented the presence of BBS-like IR in the central nervous system of fish. In the cartilaginous fish *S. canicula*, BBS-like IR material was reported in ventral forebrain regions of the telencephalon and hypothalamic preoptic area, as well as in the habenular complex (Vallarino *et al.*, 1990). In this fish species, BBS-like IR was also described in a prominent nerve tract within the infundibular floor and in the median eminence, where IR-fibres appeared to form close contact with the vascular system of the pituitary portal system, suggesting a neuroendocrine role for BBS-like peptides (Vallarino *et al.*, 1990). No studies have examined the presence of BBS/GRP-like IR in the fish pituitary.

1.5.1.2 Mammals

Studies indicate that BBS/GRP-like peptides are widely distributed within neurons of the mammalian gastrointestinal tract. In the rat, BBS/GRP-like IR has been described in high concentrations within the gastric mucosal and muscle layers, as well as within nerve fibres of the intestinal mucosa (Dockray *et al.*, 1979). Greeley *et al.* (1986) compared the distribution of BBS/GRP-like peptides within several mammalian species and reported that BBS/GRP-like IR was present in nerve fibres throughout the entire gastrointestinal tract of the rat, dog, and human; GRP-27 and GRP-10 activity was greatest in the colon of the rat, while the dog and human exhibited highest concentrations of GRP-27, GRP-10, and BBS in the fundic mucosal layer.

BBS/GRP-like peptides have also been documented in the mammalian central nervous system and in particular, in brain areas associated with the regulation of feeding behavior. Moody *et al.* (1980) reported BBS/GRP-like IR within synaptosomal fractions of rat brain. When compared to levels in the cerebellum, the density of BBS/GRP-like peptides were 30-fold greater in the substantia gelatinosa, nucleus tractus solitarius (NTS), amygdala and within hypothalamic nuclei. High densities of BBS/GRP-IR containing nerve terminals and axons have also been described in several brain regions including specific hypothalamic nuclei, the periventricular nucleus of the thalamus, lateral parts of the interpeduncular nucleus, the dorsolateral tegmental nucleus, and the dorsal nucleus of the vagal nerve (Panula *et al.*, 1982). Perikarya containing BBS/GRP-like IR have been detected in the dorsolateral tegmental nucleus of the pons, the NTS, the dorsal parabrachial nucleus, the lateral reticular nucleus, the anterior part of the interpeduncular nucleus, and the paraventricular nucleus (PVN) within the hypothalamus (Panula *et al.*, 1982). Cell bodies and nerve fibres containing BBS/GRP-like IR have also been localized in the suprachiasmatic nucleus (SCN) of the hamster (Reuss, 1991). BBS/GRP-like IR material has also been described in the anterior and the intermediate and/or posterior pituitary lobes of several mammalian species, including the rat, guinea pig, cat, dog, pig, cow, monkey, and human (Houben and Denef, 1991a; Steel *et al.*, 1992).

1.5.2 Bombesin and Feeding Behavior

1.5.2.1 Fish

With the exception of one preliminary report whereby BBS was described to decrease food intake in carp (Beach *et al.*, 1988), no studies have examined in detail the role of neuropeptides in the regulation of feeding behavior in any lower vertebrate. However, the effects of BBS on feeding-related activities, such as alterations in gut motility, gastric acid secretion and visceral activity, have been documented in several fish species, including the rainbow trout *O. mykiss*, the sea scorpion *Myoxocephalus scorpius*, the Atlantic cod *G. morhua*, the daddy sculpin *Cottus scorpius*, and the spiny dogfish *S. acanthias* (Holmgren *et al.*, 1982; Thorndyke and Falkmer, 1982; Holmgren and Jonsson, 1988; Thorndyke and Holmgren, 1990). In *G. morhua*, BBS/GRP-like peptides stimulate contractility of longitudinal and circular stomach muscles (Holmgren and Jonsson, 1988). This effect is potentiated by acetylcholine in *O. mykiss*, *G. morhua* and *C. scorpius* (Thorndyke and Falkmer, 1982; Thorndyke and Holmgren, 1990). BBS-induced increases in gut contractility result in a compression of the vasculature within the stomach muscle wall. This in turn limits the blood flow delivered from the stomach and may be one mechanism whereby humoral signals are altered during feeding to induce satiety. In *S. acanthias*, Bjenning *et al.* (1990) reported that blood flow in isolated, perfused stomach tissues was altered following the administration of BBS. Initially, there was an increase in blood flow rate followed by a larger decrease of blood flow. Eventually a slower, long lasting increase in flow rate occurred.

Presently, no studies have investigated the central effects of BBS with respect to feeding behavior in fish. However, one early study by Kavaliers and Hawkins (1981) reported that BBS injection into the brain of the white sucker, *Catostomus commersoni*, causes fish to alter their behavior with respect to thermoregulation and the selection of ambient water temperature.

1.5.2.2 Mammals

In mammals, it is well established that peripherally administered BBS results in a suppression of food intake, accompanied by the classic sequence of behaviors associated with satiety such as grooming, sniffing, eventual withdrawal from the food site, and rest

or sleep (Gibbs *et al.*, 1979; Avery and Livosky, 1986; for review McCoy and Avery, 1990). Early studies by Gibbs *et al.* (1979) demonstrated a suppression in liquid food intake within 15 minutes of ip BBS injection. In this study, several other observations indicated that BBS was acting as a satiety signal. BBS was ineffective in preventing or slowing the early phase of feeding; rather, BBS enhanced satiety by shortening meal duration. In addition, no alternate abnormal behaviors were noted, demonstrating the specificity of BBS actions on feeding. Finally, high doses of BBS failed to produce illness or distress, further supporting a physiological role of peripheral BBS in the regulation of feeding behavior.

Other more recent studies have shown that peripheral injection of BBS can reduce food intake based on dietary preferences. In food-deprived rats, Avery and Livosky (1986) described a significant decrease in protein intake at 30 and 60 minutes following BBS administration. McCoy *et al.* (1990) similarly reported a selective dose-dependent suppression in protein intake within 30 minutes following BBS injection. Such actions by peripherally administered BBS may occur through several mechanisms. BBS may alter gastrointestinal motility and intragastric pressure via specific, high affinity receptors within the gastric muscle cells (Falconiere *et al.* 1988, Margolis *et al.*, 1989; Severi *et al.*, 1991). Altered stomach contractility by BBS may also contribute to peripherally-induced BBS feeding suppression (Deutsch, 1980). Ladenheim and Ritter (1991) reported that peripheral BBS also acts to induce suppressed feeding through the activation of small, unmyelinated, sensory neurons.

BBS appears to suppress feeding behavior through predominantly direct actions within the central nervous system. Stuckey and Gibbs (1982) have reported that bilateral injections of BBS (5 -100 ng) into the lateral hypothalamus of food deprived rats decreases feeding, while Willis *et al.* (1984) also described suppressed food intake following BBS injection into specific brain regions. Similarly, Kyrkouli *et al.* (1987) demonstrated that microinjection of BBS into medial and lateral hypothalamic regions resulted in a suppression of food intake. Recently, Merali *et al.* (1993) reported that blockade of brain BBS/GRP receptors by a specific BBS/GRP antagonist results in an increase in food intake in satiated rats.

Regions of the mammalian hindbrain also appear to be involved in BBS-mediated feeding suppression. Notably, the NTS of the hindbrain, which receives peripheral

information and inputs signals to the PVN, has been shown to contain a high density of BBS-like binding sites (Zarbin *et al.*, 1985), BBS-like IR (Panula *et al.*, 1982; Panula, 1986), and BBS mRNA (Wada *et al.*, 1990). Infusion of BBS into the NTS results in a 63% decrease in food intake in rats (DeBeaurepaire and Suaudeau, 1988), further supporting this brain area as a site for BBS actions. In line with this, Flynn (1991) demonstrated that fourth ventricle injections of low doses of BBS (5, 10, and 20 ng) resulted in a significant decrease in fluid intake. These actions were specific, since pre-injection of rats with the BBS receptor antagonist [D-Phe^{12}, Leu^{14}] -BBS blocked the effects of BBS on feeding behavior.

Another mechanism whereby central BBS may act to modulate feeding behavior is through the regulation of peripheral gut visceral activity during food intake. Spencer and Talman (1987) reported that microinjection of BBS into the NTS resulted in a dose-dependent increase in tonic gastric and phasic intraluminal pressure. Intracerebroventricular injection of BBS also results in a significant decrease in gastric fluid volume, a decrease of HCl output, a significant increase in pH, and an elevation in serum immunoreactive gastrin (Tsalis *et al.*, 1990).

1.5.3 Bombesin and Growth Hormone

1.5.3.1 Bombesin Regulates GH Release Directly at the Level of the Pituitary

Studies which demonstrate that BBS/GRP-like peptides alter circulating serum GH levels have been conducted only in mammals (Kabayama *et al.*, 1984; Kentroti and McCann, 1985). Such BBS-mediated GH actions may result from direct effects of BBS at the level of the pituitary somatotropes, since evidence indicates that BBS/GRP-like peptides can directly modify pituitary GH release from cultured pituitary cells *in vitro*. Westendorf and Schonbrunn (1982) demonstrated an acute release of GH from pituitary GH_4C_1 cells following incubation with 0.5 nM BBS, a dose comparable to endogenous circulating levels of BBS-like peptides measured in rat plasma (Brown *et al.*, 1978). Similarly, Kentroti and McCann (1985) reported a dose-dependent stimulation of GH release following incubation of cultured pituitary cells with 10^{-9} to 10^{-6} M GRP. The GH responsiveness to BBS appears to be additive in the presence of estradiol (Houben

et al., 1990). Furthermore, BBS/GRP peptide actions on the pituitary somatotropes appear receptor specific, since the BBS/GRP receptor antagonist L 686, 095-001C002 blocks GRP-mediated GH release (Houben and Denef, 1991b). The presence of [^{125}I]Tyr$_4$-BBS-binding sites on GH cells from the rat anterior pituitary have been documented, supporting a regulatory role for BBS on pituitary GH release (Wouters, G., Andries, and Denef, C. unpublished observations; through Houben and Denef, 1991b).

Current studies indicate that when administered both *in vivo* and *in vitro*, BBS/GRP-like peptides can also alter pituitary GH secretion through an interaction with the hypothalamic GH regulators, GRF and SRIF. Kabayama *et al.* (1984) reported that while intravenous injection of GRF into rats causes a dose-dependent increase in plasma GH, third brain ventricle injection of GRP nearly abolishes this GRF-induced GH response. Such inhibitory effects produced by GRP are attenuated when rats are pretreated with anti-SRIF, suggesting the involvement of hypothalamic SRIF in GRP-mediated GH actions. Kentroti *et al.* (1988) also demonstrated a dose dependent increase in the release of SRIF following incubation of median eminence fragments with 10^{-10} to 10^{-6} M GRP.

1.5.4 Bombesin Receptors

BBS-induced alterations in feeding behavior, thermoregulation, locomotion, and anterior pituitary hormone secretion appear to be mediated through specific membrane-bound receptors (for review Spindel *et al.*, 1993). Putative BBS/GRP-like receptors have been characterized in both the peripheral and central nervous systems (Moody *et al.*, 1978; Westendorf and Schonbrunn, 1983; Houben and Denef, 1991a; Vigna *et al.*, 1987), in pancreatic acinar cells (Jensen *et al.*, 1978; Swope and Schonbrunn, 1987), antral gastrin cells (Vigna *et al.*, 1987; Vigna *et al.*, 1989; Vigna *et al.*, 1990), human small cell lung carcinoma cell lines (Moody *et al.*, 1985) and Swiss 3T3 cells (Sinnett-Smith *et al.*, 1990).

Binding studies have revealed that BBS/GRP-like peptides bind to a single class of high affinity receptors located in the plasma membrane (Jensen *et al.*, 1978; Moody *et al.*, 1985; Millar and Rozengurt, 1990; Sinnett-Smith *et al.*, 1990; Vigna *et al.*, 1990). These receptors have been cloned from mouse, rat, and human cells (Giladi *et al.*, 1993). Additionally, it has been reported that BBS binds to a second receptor site

specific for neuromedin B-like peptides; these sites are localized in the rat esophageal muscularis mucosa (Von Schrenck *et al.*, 1989). Severi *et al.* (1991) described two binding sites for BBS-related peptides; one receptor subtype has a high affinity for BBS, GRP and the antagonist Ψ13, 14-BBS, and a low affinity for neuromedin B (BBS/GRP receptor), whereas the second subtype has a high affinity for neuromedin B, a moderate affinity for Ψ 13, 14 BBS, and very low affinity for BBS and GRP (neuromedin B receptor). This supports *in vitro* studies with respect to the biological effects of BBS and the BBS-related peptides belonging to the ranatensin-litorin subfamily, where BBS is three fold more potent than neuromedin B at contracting isolated gastric muscle cells, where the BBS/GRP receptor subtype is located (Severi *et al.*, 1991).

Several classes of BBS antagonists have been synthesized. Substance P derivatives have low potency and lack specificity, while BBS derivatives have low potency with better specificity. BBS derivatives with a reduced peptide bond and GRP derivatives containing an amino-terminal ester and a C-terminal amide or alkyl ether have also been synthesized. These latter classes are also variable in their antagonistic actions. For example, one of the most potent BBS antagonist analogues [Leu^{13},Ψ(CH_2NH) Leu^{14}]-BBS is successful in inhibiting BBS-stimulated pancreatic amylase secretion *in vivo* (Alptekin *et al.*, 1991), but fails to inhibit GH release *in vivo* following brain injection (Houben and Denef, 1990) and may be partially agonistic on GH and prolactin release when examined *in vitro* following incubation with pituitary cell aggregates (Houben and Denef, 1991b).

The presence of BBS/GRP-like binding sites within the central nervous system of lower vertebrates has not been documented. However in preliminary studies, Vigna and Thorndyke (1989) reported ^{125}I-labelled BBS binding sites in longitudinal and circular muscles of the antral stomach in the teleost, *Scorpaeichthys marmoratus*.

1.6 Cholecystokinin

1.6.1 Characterization and Distribution

Cholecystokinin (CCK), gastrin, and the amphibian skin peptide caerulein, belong to a family of peptides characterized by sharing an identical biologically active carboxyl terminal pentapeptide sequence (-Gly-Trp-Met-Asp-Phe-NH_2) (Table 1.2) (Dockray,

Table 1.2. The amino acid sequence of the carboxy termini of CCK/gastrin-like peptides.

CCK-33-s	Asp-Tyr(SO_3H)-Met-Gly-Trp-Met-Asp-Phe-NH_2
CCK8-s	Asp-Tyr(SO_3H)-Met-Gly-Trp-Met-Asp-Phe-NH_2
CCK4	Trp-Met-Asp-Phe-NH_2
Gastrin17-s	Glu-Glu-Asp-Tyr(SO_3H)-Gly-Trp-Met-Asp-Phe-NH_2
Caerulein	Glp-Gln-Asp-Tyr(SO_3H)-Thr-Gly-Trp-Met-Asp-Phe-NH_2

1976, Vanderhaeghen *et al.*, 1980). Members of the CCK/gastrin family of peptides are known to be present throughout the vertebrates (Dockray, 1976; Crim and Vigna, 1983; Dockray and Dimaline, 1984), and it has been proposed that discrete CCK and gastrin molecules may have arisen from an ancestral CCK/caerulein-like peptide at the evolutionary level of divergence of amphibians and reptiles (Larson and Rehfeld, 1977; for review Vigna, 1994). In immunohistochemical studies involving CCK/gastrin-like peptides, it has been difficult to distinguish between distinct CCK and gastrin-like molecules, since antisera directed to the highly conserved carboxyl -terminal pentapeptide sequence contained within both peptides displays unavoidable cross-reactivity with all CCK/gastrin-like peptides of 5 amino acids or greater. Despite this limitation, immunohistochemical studies in lower vertebrates, where the N-terminal sequence of CCK is unknown, can be accomplished through use of antisera directed to the CCK/gastrin carboxyl-terminal. Studies using such antisera have revealed a widespread distribution of CCK/gastrin-like IR material within the central and peripheral nervous systems of teleost fish.

1.6.1.1 Fish

In fish, it has been suggested that the CCK/gastrin molecule resembles CCK more than the sequence of gastrin, suggesting that CCK evolved earlier than gastrin (Crim and Vigna, 1983; Vigna, 1994). However, in the goldfish brain the presence of CCK-like peptides which are distinguishable from gastrin by bioassay has been described (Sankaran *et al.*, 1987). Slight differences in the capacity of CCK8 sulfated (CCK8-s) and gastrin 17 sulfated to stimulate the release of GH from goldfish pituitary fragments *in vitro* have also been reported (Himick *et al.*, 1993; Chapter 6). Additionally, differences between the actions of the sulfated forms of CCK8 and gastrin 17 in stimulating contractility of isolated gallbladder strips from the rainbow trout, *O. mykiss* have been documented (Aldman and Holmgren, 1987). Even in the more primitive cyclostomes, *Lampetra tridentata* and *Lampetra fluviatilis*, the presence of CCK-like molecules distinguishable from gastrin fragments have been reported following gel chromatography of intestinal extracts (Holmquist *et al.*, 1979). In addition to the possible presence of distinct CCK and gastrin molecules in teleosts, heterogeneity in the

number of CCK-like peptides may also be present (Vigna *et al.*, 1985).

In teleosts, studies indicate that CCK/gastrin-like peptides are widely distributed within the gastrointestinal tract. Endocrine cells of the gut have been reported to contain CCK/gastrin-like IR in a number of fish species, including the Atlantic cod *G. morhua* (Larsson and Rehfeld, 1978; Jonsson *et al.*, 1987), cyprinids such as *Barbus conchonius* (Rombout and Taverne-Thiele, 1982) and *Leuciscus idus melanotus* (Burkhardt-Holm and Holmgren, 1989), the guppy *P. reticulata*, the catfish *Corydoras schultzei* (Langer *et al.*, 1979), and a salmonid *O. mykiss* (Holmgren *et al.*, 1982). Nerve fibres immunoreactive for CCK/gastrin-like peptides have also been described in the stomach, intestine and/or rectum of several fish, including the carp *C. carpio* (Bjenning and Holmgren, 1988), *P. reticulata* and *L. idus* (Langer *et al.*, 1979), and the sea scorpion, *N. scorpius* (Reinecke *et al.*, 1981; Bjenning and Holmgren, 1988).

CCK/gastrin-like IR perikarya and IR nerve fibres have also been described within the teleost central nervous system. In *O. mykiss*, CCK/gastrin-like IR material has been documented in the nucleus lateralis tuberis (NLT), the hypophysial stalk, and the proximal pars distalis (PD) of the anterior pituitary (Notenboom *et al.*, 1981). Similarly, in *P. latipinna* CCK/gastrin-like IR fibres have been described in regions of the rostral and caudal telencephalon and in hypothalamic perikarya and fibres including the NLT, nucleus entopeduncularis (NE), nucleus preopticus (NPO), nucleus anterioris tuberis (NAT), and the lateral recess of the inferior lobe (Batten *et al.*, 1990). In this fish species, CCK/gastrin-like IR has also been localized in the proximal PD, midbrain, nucleus habenularis (NH) of the diencephalon, and within the thalamic nucleus dorsomedialis (DMN) and nucleus ventromedialis (VMN). CCK/gastrin-like IR fibres and IR cell bodies and specific binding sites for [^{3}H]-CCK have also been described in the sea bass *Dicentrarchus labrax* ventral telencephalon, preoptic region, caudal hypothalamus, optic tectum, and thalamus (Moons *et al.*, 1992). CCK/gastrin-like IR fibres have also been reported in the PD of *D. labrax* (Moons *et al.*, 1988), and in the proximal PD, and to a lesser extent, within the rostral PD of the goldfish, *C. auratus* (Himick *et al.*, 1993; Chapter 6). In the goldfish, CCK/gastrin-like IR fibres were found to be co-distributed amongst the somatotropes and gonadotropes (Himick *et al.*, 1993; Chapter 6).

1.6.1.2 Mammals

In mammals, multiple forms of CCK peptides, including CCK58, CCK39, CCK33, CCK22, CCK12, CCK8 and CCK4, have been reported peripherally within nerves and endocrine cells associated with the gastrointestinal tract and within the central nervous system (for review Beinfeld, 1988). Such heterogeneity in these CCK peptide forms may be the result of differential post-translational processing of the single gene product, proproCCK, since all forms appear to be derived from a single gene (Deschenes *et al.*, 1984; Turkelson and Solomon, 1990). Not all species contain all CCK peptides and controversy surrounding the real circulating forms present within an organism stems from the actual extraction procedures used, where the longer forms of CCK peptides are more susceptible to degradation (Turkelson and Solomon, 1990).

Using antisera specific for the N-terminal regions of either the CCK or gastrin molecules, several studies have mapped the distribution of CCK-like and gastrin-like IR in the mammalian peripheral and central nervous systems (for review Beinfeld, 1988). In the guinea pig and rat gut, CCK-like IR has been reported in the myenteric plexus of Auerbach and in the submucous plexus of Meissner, while several perikarya of Meissner's plexus also contain IR material (Larsson and Rehfeld, 1979; Schultzberg *et al.*, 1980). Nerve fibres containing CCK-like IR have also been demonstrated in the lamina muscularis mucosal and mucosal layers (Larsson and Rehfeld, 1979; Hutchinson *et al.*, 1981). Additionally, IR fibres have been described in the coeliac-superior mesenteric ganglion complex (Larsson and Rehfeld, 1979; Rehfeld *et al.*, 1979). The presence of CCK4 (or tetragastrin-like) -IR has also been reported in pancreatic nerve terminals surrounding islet cells, indicating that CCK peptides regulate pancreatic hormone secretion. CCK-like IR has also been localized in specific mucosa I-cells of the duodenum and proximal jejunum (Polak *et al.*, 1975; Larsson and Rehfeld, 1979). Using antisera directed to the N-terminal of gastrin, gastrin-like IR has been localized in afferent vagal and sciatic nerve fibres, and in nerves of the muscular wall of the proximal colon originating in the vagus nerve (Uvnas-Wallensten *et al.*, 1977; Larsson and Rehfeld, 1978). Gastrin-like IR has also been reported in G-cells of the antrum and in IG-cells of the proximal small intestine (Buchanan *et al.*, 1979).

Nerve fibres containing CCK-like IR are widely distributed within the mammalian

central nervous system, with high densities of IR material localized in neurons of the hippocampus, nucleus accumbens, caudate nucleus, cerebral cortex, hypothalamus, spinal cord and colon (Dockray 1976; Larsson and Rehfeld 1979; Beinfeld *et al.*, 1980; 1981; Vanderhaeghen *et al.*, 1980). Within the hypothalamus, CCK-like IR has been described in perikarya of both the supraoptic nucleus and the PVN, and it has been suggested that these cells are the origin of CCK-like IR material located within the posterior pituitary and in the median eminence (Kiss *et al.* 1985).

1.6.2 Cholecystokinin and Feeding Behavior

1.6.2.1 Fish

Although no studies have investigated the role of CCK in the regulation of feeding behavior in fish, several reports have indicated that members of the CCK/gastrin family of peptides are capable of altering the mechanical events associated with the feeding. In isolated gallbladder muscle strips of the coho salmon, *O. kisutch*, the sulfated forms of both CCK and gastrin are equipotent in stimulating contraction, whereas the nonsulfated forms of these peptides are approximately 1000 times less potent (Vigna and Gorbman, 1977). In line with this, Rajjo *et al.* (1988) described an increase in gallbladder contraction *in vitro* in the bluegill *L. macrochirus*, and the killifish *Fundulus heteroclitus*, following application CCK8-s or sulfated caerulein to the tissue chambers. The nonsulfated forms of these peptides were less potent in their stimulatory capacity. In the salmonid *O mykiss*, increased contraction of isolated gallbladder muscle has similarly been described following challenge with sulfated CCK, caerulein, and the nonsulfated form of CCK (Aldman and Holmgren, 1987). Other studies by Jonsson *et al.* (1987) have demonstrated that isolated gastrointestinal smooth muscle strips from *G. morhua* exhibit increased contractility when exposed to caerulein, and the sulfated forms of CCK and gastrin. Together these studies indicate that CCK/gastrin-like peptides likely play a fundamental role in regulating short-term events associated with the digestive phase of feeding in fish. One recent study by Burkhardt-Holm and Holmgren (1989) examined the long-term influence of starvation on CCK/gastrin-like IR in intestinal nerves and enteroendocrine cells of *P. reticulata* and *L. idus melanotus*. After more than 6 weeks, no alterations were observed in either the appearance or the amount of nerve and endocrine cell IR material.

1.6.2.2 Mammals

A number of studies in mammals indicate that CCK acts at both peripheral sites within the gastrointestinal tract and in the brain to initiate satiety. In response to a meal, CCK peptides are released by the gut which then bind to receptors located on ascending vagal fibers. These neurons then form synapses in the caudal nucleus of the NTS and input signals to the ventral medial hypothalamus (VMH) and the PVN, otherwise known as the "satiety centers" of the brain (Miceli, 1985; Morley, 1987; Beinfeld, 1988; Bado *et al.*, 1989). Simultaneously, CCK is released in the brain and then binds to its respective receptors and acts within the brain to terminate feeding (for review Baile and Della Fera, 1988; Sakatani *et al.*, 1987; Reidelberger, 1989 for review Silver and Morley, 1991).

Administration of CCK into the brain has confirmed the satiation actions mediated by this peptide. In two-hour fasted sheep, continuous injections of low doses of CCK8-s into the lateral ventricles reduced food intake by 56% within 30 minutes (Baile and Della-Fera, 1988). On the contrary, CCK antisera injected into the lateral ventricles of sheep results in an increase in feeding (Della-Fera *et al.*, 1981). Similarly, infusions of CCK8-s into the third and lateral cerebral ventricles of the dog suppresses feeding (Sakatani *et al.*, 1987; Inui *et al.*, 1988). Faris *et al.* (1983) also described decreased food intake in the rat following injection of CCK into the hypothalamic PVN, whereas high doses of the nonsulfated form of CCK had no effect on feeding when injected into the lateral ventricles of sheep. Specific brain CCK receptor antagonists have been shown to postpone satiety (Dourish *et al.*, 1989).

Peripheral administration of CCK8-s also alters feeding behaviour. In the cat, systemic infusion of CCK8-s induces short-term transient satiation (Bado *et al.*, 1989), while in man intravenous CCK8-s injection (4.6 ng/kg/min) causes a decrease in both solid and liquid food intake (Stacher, 1985). Raybould *et al.* (1988) have reported that peripheral CCK8-s acts to suppress feeding through CCK activation of vagal mechanoreceptive endings in the gastric corpus wall; CCK-stimulated vagal afferents then input signals to satiety centers in the brain via the NTS. In support of this, Morley (1987) has shown that in the rat, lesions applied to either the NTS or the PVN inhibit peripherally-mediated CCK actions in feeding suppression. Specific peripheral type

CCK receptor antagonists (L 364,718 or MK-329) have been shown to produce a dose-related increase in feeding in humans (Wolkowitz *et al.*, 1990) and pigs (Ebenezer *et al.*, 1990).

1.6.3 Cholecystokinin and Growth Hormone

1.6.3.1 Cholecystokinin Regulates GH Release

Limited studies in mammals indicate that CCK administration alters circulating plasma GH levels. A significant increase in plasma GH has been reported at 15 min post-intravenous injection of CCK8-s into the rat (Vijayan *et al.*, 1979), while Matsumura *et al.* (1984) have demonstrated an increase in plasma GH within 20 minutes of iv injection of CCK8-s. Such changes in circulating levels of GH may be the result of a direct action of CCK at the level of the pituitary. Morley *et al.* (1979) reported an acute release of GH from anterior rat pituitary fragments following incubation with CCK8-s, while treatment of GH_3 pituitary tumor cells with CCK8-s has been reported to reverse the inhibitory effects of SRIF on GH. Similarly, Matsumura *et al.* (1984) described a dose-dependent release of GH *in vitro* following incubation of pituitary fragments with 10^{-11}M to 10^{-7}M CCK8-s.

CCK also acts to modify the secretion of another anterior pituitary hormone, gonadotropin (GtH). In adult female rats, the regular 4 day cyclic pattern of gonadal steroid secretion during estrous corresponds with the rhythmic variation in both GtH secretion and food intake. Micevych *et al.* (1988) reported that estrous cycle variations also occur in the brain with respect to CCK and the binding of sulfated ^{125}I-CCK8-s in the VMH. These authors suggest that the VMH is an important site of interaction between estradiol (E_2) and CCK, and that E_2 promotes the release of CCK in regions of the hypothalamus. Other studies demonstrate that a relationship between CCK and GtH exists. Injection of CCK8-s into the third ventricle of the rat results in an acute significant decrease in plasma luteinizing hormone (LH) (Vijayan *et al.*, 1979), while infusion of CCK into the medial preoptic area of the rat enhances the secretion of LH-releasing hormone (LH-RH) (Kimura *et al.*, 1983). Such a relationship between CCK and GtH may exist in lower vertebrates, since Doerr-Schott *et al.* (1979) have reported the presence of CCK/gastrin-like activity in the infundibularis ventralis of the caudo-ventral hypothalamus (the GtH releasing-hormone (GnRH) producing centre) in the

clawed toad *Xenopus laevis*.

1.6.4 Cholecystokinin Receptors

Studies characterizing the CCK receptor have reported two or possibly three classes of CCK/gastrin binding sites in several mammalian species, including the rat, mouse, guinea pig, and dog (Sankaran *et al.*, 1980; 1982; Altar and Boyar, 1989; Yu *et al.*, 1990). The first class of binding sites, the CCK-B (brain) receptor subtype, interacts nearly equally well with all forms of sulfated and nonsulfated CCK and gastrin, and exhibits a widespread distribution throughout the central nervous system (for review Vigna, 1994). The gastrin receptor is very similar to, if not identical with the CCK-B receptor (Pisegna *et al.*, 1992), and it may in fact be that these two binding sites represent only one CCK-B/gastrin receptor (for review Vigna, 1994). While species differences are evident with respect to the densities of this receptor type in specific brain regions (Niehoff, 1989), CCK-B receptors are consistently distributed in the limbic, olfactory, visual and cortical areas (Saito *et al.*, 1980; Finkelstein *et al.*, 1983). The presence of CCK receptors have also been described in the cerebellum of only the guinea pig (Williams *et al.*, 1986; Niehoff, 1989). At present, the function of these cerebellar receptors remains unknown; significant amounts of CCK-IR have not been observed within this brain area (Larsson and Rehfeld, 1979). The CCK-B receptor has recently been cloned from the rat brain (Wank *et al.*, 1992).

The second class of mammalian CCK binding sites, the CCK-A (alimentary) receptor subtype, has a strict requirement for CCK peptides containing a sulfated tyrosine residue at position seven from the carboxyl terminus (for review Vigna, 1994). For example, it has been shown that the affinity of pancreatic acini receptors for CCK8-s is approximately 5000 times greater than that for the nonsulfated forms of either CCK8 or gastrin (Sankaran *et al.*, 1980). CCK-A receptors occur mainly in the periphery within organs and endocrine cells associated with the gastrointestinal tract, such as pancreatic acinar cells, pancreatic membranes, and gallbladder muscle (Sankaran *et al.*, 1980; Innis and Snyder, 1980; Morini *et al.*, 1990), but also in highly localized brain regions, such

as the NTS (Moran *et al.*, 1986). CCK-A receptors have also been localized on the vagus nerve; these binding sites likely mediate the well established satiety effects mediated by peripheral CCK (Innis and Snyder, 1980). The CCK-A receptor has recently been cloned from the rat pancreas (Wank *et al.*, 1992).

Antagonists with high degrees of relative specificity for CCK-A and CCK-B have been produced and their effects on *in vivo* actions, such as feeding behavior, have been investigated (Moran *et al.*, 1986; Dourish *et al.* 1989). Dourish *et al.* (1989) reported that CCK-B antagonists were more potent and efficacious than CCK-A antagonists in stimulating feeding behavior in satiated rats. This, however, contrasts with findings of the pharmacological specificity of actions of exogenously administered CCK receptor antagonists, where blockade of CCK-A, but not CCK-B, postpones satiety in the rhesus monkey (Moran *et al.*, 1993). Specific antagonists for the CCK-A receptor include devazipide, while L-365, 260 and PD134308 are selective for CCK-B/gastrin receptors.

According to Vigna (1994), CCK-A and CCK-B receptors of endotherms may have originated from a single "primitive" CCK (CCK-P) receptor which is still present in ectotherms. In contrast to CCK-A and CCK-B/gastrin receptors, the CCK-P binding site interacts with high affinity only with sulfated forms of CCK and gastrin (Vigna *et al.*, 1986). In other words, the positioning of the sulfated tyrosine from the carboxyl terminal is not a strict requirement for binding. It is suggested that the CCK-A and CCK-B/gastrin receptors of endotherms evolved from CCK-P at the phylogenetic evolutionary level of the birds.

Only one study to date has documented the presence of CCK/gastrin binding sites in the teleost central nervous system. In the sea bass *D. labrax*, Moons *et al.* (1992) reported specific binding sites for [^{3}H] -CCK in the dorsal and ventral telencephalon, the preoptic, tuberal and posterior hypothalamus, optic tectum, and the valvulla cerebelli. Specific binding was also detected in the dorsal medulla oblongata just ventral to the vagal lobe at the level of the facial and vagal motor nuclei. No antagonist is currently available which selectively acts on the ectothermic CCK-P receptor (Vigna, 1994).

1.7 Summary

Overall, the literature presently reviewed demonstrates that: 1) fish regulate the amount of food they consume, and that a feeding center exists in the teleost brain, 2) GH may participate in either the regulation of feeding behavior or become altered as a consequence of feeding, 3) the neuropeptides, BBS and CCK, act through specific, high affinity binding sites to suppress feeding behavior and release anterior pituitary hormones in mammals, and 4) several fish species possess BBS/GRP-like IR peptides and CCK/gastrin-like IR peptides in the central nervous system.

With this information, I proposed the hypothesis that BBS- and CCK-like peptides are present in the goldfish central nervous system where they act through specific, high affinity binding sites to regulate feeding behavior and anterior pituitary hormone secretion. This hypothesis forms the basis of my research presented in this thesis.

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Chapter 2

Feeding Behavior in Goldfish

2.1 Introduction

Little information exists with respect to how goldfish regulate the amount of food that they consume, although the behavioral events which accompany feeding in cyprinids have been documented (for reviews Lammens and Hoogenboezem, 1991; Sibbing, 1991). According to Holling (1966) and Sibbing *et al.* (1986), the feeding actions of cyprinids in general can be classified as follows: 1) search, 2) detection, 3) pursuit, 4) intake, 5) taste selection, 6) size selection, 7) transport, 8) mastication, 9) swallowing, and 10) digestion. Depending on the food type and fish species, some of these actions may dominate whereas others may play a minor role. In the goldfish, food intake and taste selection are predominant activites associated with feeding behavior. During food capture and intake, these fish take in food particles through a slow suction motion characterized by both particulate feeding (fish protrude the upper jaw and aim their circular gape towards the particle) and gulping (water and food are slowly sucked in) (Sibbing, 1991). Following food intake, goldfish decide if the food is palatable or not through a taste selection process. This includes several behaviors such as "spitting" (when food is found to be undesirable), or selective retention (when food is palatable) (Sibbing, 1991). In goldfish, the extremely well developed vagal lobes indicate the importance of taste perception within the oropharyngeal system during feeding (Morita and Finger, 1987; Finger, 1988).

Studies have shown that goldfish precisely regulate the amount of food that they consume on a daily basis (for review Chapter 1). Early experiments by Rozin and Mayer (1961; 1964) reported that conditioned goldfish distribute their feeding responses fairly evenly over time within the light phase. In addition, these fish accurately regulate food consumption based on ambient temperature, and the caloric and nutrient content present in their food. Mechanical events associated with digestion, such as gastric or anterior intestinal distention, and feed evacuation rates, gastrointestinal motility and gut emptying all contribute to how much food fish will consume (for review Holmgren *et al.*, 1983;

Weatherley and Gill, 1987). In turn, these mechanical events are regulated by neuropeptides and neurotransmitters (for reviews Fange and Grove, 1979; Holmgren *et al.*, 1983). Additionally, neural areas within the teleost brain have been shown to regulate the amount of food consumed (for reviews Peter, 1979; Demski, 1981; 1983). It is likely that both neuropeptides and hormones play key central roles in the regulation of feeding behavior in fish.

Experiments in this chapter are based on two main objectives. To investigate the role that neuropeptides play in feeding behavior in fish, a feeding protocol was developed whereby food intake in goldfish could be accurately measured following experimental manipulation. The results from these experiments formed the basis of future studies in other chapters, where the effects on feeding behavior of two neuropeptides, cholecystokinin and bombesin, were examined. The second objective in this chapter was to investigate the acute changes in growth hormone (GH) that occur during feeding in goldfish, since GH has been implicated in the regulation of food intake in mammals (refer to Chapter 1). Results from studies in this chapter were used to interpret the short-term changes observed in circulating serum GH levels following neuropeptide mediated feeding suppression in subsequent chapters (Chapters 3 and 6).

2.2 Materials and Methods

Experimental Animals

Male and female goldfish (20 to 40 g) of the common or comet varieties, were purchased from Ozark Fisheries (Stoutland, MO) at several times of the year. Fish were classified into sexually gonadal phases based on morphological characteristics of the gonad and the gonadosomatic index (GSI; gonad weight as a percentage of total body weight x 100%) as described by Wong (1993). Briefly, male and female fish in the gonadal regressed stage contained thin, grayish colored gonads and GSI was less than 1% (May to November). In early and late gonadal recrudescing female fish, ovaries were grayish-green in color and GSI ranged from 1.5% to 8% (December to March). Female goldfish were considered sexually mature when GSI exceeded 8%, and ovaries contained visible vitellogenic oocytes (March to April). Male goldfish were considered gonadal early and late gonadal recrudescent when GSI ranged from 1.5% to 3%, and were sexually mature when GSI was greater than 3%. Fish were maintained under simulated

Edmonton natural photoperiod in 1.8 kL tanks which received a constant flow of aerated and dechlorinated city water at 17 ^{O}C and were fed *ad libitum* at least once daily with commercially prepared trout pellets (Trout production 3/32 or 5/32; Rangen Inc., protein 40%, fat 12%, ash 15%, fiber 5%, mineral 2%) until use in either observational or group feeding experiments.

Observational Experiments

Prior to an experiment, goldfish were anesthetized with 0.05% tricaine methanesulfonate (TMS: Syndel laboratories, Vancouver, BC), weighed to the nearest 0.1 g, and randomly assigned to 65 L observatory aquaria containing flow-through, aerated water at 17 - 19^{O} C (3 fish per aquarium; photoperiod 16L:8D). Fish were identified by individual markings. All test aquaria contained gravel substrate and floating synthetic weeds, while tank sides were covered by opaque barriers to minimize external disturbances. Goldfish were fed once daily a 2% wet body weight (bw) ration of floating pellets (EXSL 440, 5/32: Rangen, Inc., Buhl, Idaho (diet #1) or 3PT Fish Food Float, Martin Feed Mills Ltd., Elmira, Ontario (diet #2)) (refer to Appendix 1 for dietary composition). These pellets were used due to their adequate dietary composition, size, ability to float (which allowed for accurate food consumption measurement) and the lack of variability in size between individual pellets (ie, the number of pellets consumed could be recorded). A 2% bw ration was chosen based on a preliminary growth experiment where relative to fish fed a 1% or 3% bw ration of diet #1 daily, fish fed a 2% bw ration exhibited optimal maintenance growth over 5 weeks of feeding. Fish were acclimated under these standardized conditions for approximately 2 weeks prior to the start of an experiment. This allowed time for adjustment to a new feeding regime and pellet type.

Goldfish feeding behavior was measured via a continuous recording or "all-occurrences" recording procedure. According to Martin and Bateson (1986), this technique provides an exact and accurate record of discrete events associated with behavior by measuring true frequencies and durations, and the times at which the behavior patterns stop and start. With continuous recording, each occurrence of feeding can be recorded together with the time of occurrence. During observational experiments, only one tank of fish could be observed at a given time interval. Therefore, goldfish in

one aquarium received their prescribed ration of floating pellets in a staggered time sequence relative to fish in other aquaria during their acclimation period; this permitted comparable starvation intervals and gut emptying rates amongst fish from the different tanks. Unless otherwise stated, in all experiments fish were administered a 2% bw ration prior to recording of feeding. In some experiments where fish were injected with saline (3 µL/g) prior to receiving their prescribed ration, food was admininstered at 15 minutes post-injection. Measurement of food intake began immediately upon entry of pellets into the tank. Statistical analyses were conducted using either Student's t-test or Duncan's multiple range test. The level of significance was considered at $p \leq 0.05$, and in all experiments error bars are represented as standard error of measurement (SEM).

Group Feeding Experiments

For group feeding experiments, goldfish were anesthetized, weighed, and randomly assigned to 65 L covered tanks receiving flow-through, aerated water. Fish were fed once daily at approximately 09:30 h a 2% bw ration of diet #2. Following the experiment, fish were anesthetized and blood samples (400 µL) were taken by puncture of the caudal vasculature with a 25 gauge needle attached to a 1 mL disposable syringe. After clotting for approximately 3-4 hours at 4°C, blood samples were centrifuged, and serum was collected and stored at -28°C until pituitary hormone analyses.

Hormone Measurements and Data Analysis

Concentrations of serum GH were determined by a specific radioimmunoassay (RIA) using purified common carp GH (Marchant *et al.*, 1989) as standards and radioligand. Briefly, serum samples were incubated in a 0.08M sodium barbital buffer (pH 8.6 containing 0.5% bovine serum albumin) containing 2.5% normal rabbit serum and rabbit anti-carp GH. After 24 hours incubation at 4°C, [^{125}I]-carp GH was added to each tube and incubation was continued for an additional 24 hours at 4°C. Precipitation of the antibody-bound hormone was accomplished by addition of diluted goat anti-rabbit gamma-globulin to each tube followed by incubation overnight at 4°C. Tubes were then centrifuged, the supernatant decanted, and the radioactivity in the bound fraction was determined. All values were corrected for nonspecific binding of the labeled hormone. RIA results were analyzed using a weighted regression of a log-logit plot.

Purified carp GH was kindly donated by Dr. H. Kawauchi (School of Fisheries Sciences, Kitasato University) for use in the radioimmunoassays.

Differences in hormone levels between groups within an experiment were analyzed by either Student's t-test, Duncan's multiple range test, or in the case of experiments containing two variables, two way ANOVA with interaction followed by least-squared means. Significance was considered at $p \leq 0.05$, and in all experiments error bars are represented as SEM.

2.3 Experiments and Results

2.3.1 Goldfish Feeding Behavior

2.3.1.1 Cumulative food intake in unhandled and saline-injected goldfish To examine the cumulative food intake of goldfish over 60 minutes of feeding while fish were acclimated to the above standardized feeding and photoperiod regime, consumption of individual pellets was recorded in unhandled, regressed goldfish. Feeding data were expressed as the amount of pellets consumed / body weight / 5 minute intervals over one hour (Fig. 2-1 a). Goldfish exhibited a cumulative feeding rate of 1.1% bw/hour when fed a 2% bw ration once daily at 19°C.

To examine whether experimental manipulation alters the cumulative feeding frequency in goldfish, regressed fish were anesthetized, injected intraperitoneally (ip) with 3 µL/g of 0.9% NaCl, and placed back into their respective tanks. Following recovery at 15 minutes post-injection, a 2% bw ration was administered and cumulative food intake was recorded for 60 minutes (Fig. 2-1 b). Relative to food intake in unhandled goldfish, fish which were experimentally manipulated showed no significant alterations in the amount of food consumed when compared at 15, 30, 45, and 60 minute intervals following food entry into tanks (Student's t-test) (Fig. 2-1 c).

2.3.1.2 Effects of repeated daily handling and injection on food intake Food intake was recorded in unhandled, gonadal recrudescent fish (day 1) and on day 2, fish were anesthetized and ip injected with saline and, at 15 minutes post-injection, food intake was recorded for 60 minutes. On days 3 to 7, fish were anesthetized and ip injected with saline as on day 2 and food intake was recorded (Fig. 2-2). Repeated handling and

injection over seven days did not significantly alter the amount of food consumed (Fig. 2-2 a). The level of frequency of expelling pellets did not change following repeated saline injections (Fig.2-2 b).

2.3.1.3 Effects of daily photocycle on food intake To establish if food intake varied over the light phase of 09:30 h to 15:30 h when experiments were conducted, data from unhandled, recrudescent goldfish were pooled into three time intervals: 09:30 h - 11:30 h, 11:30 h - 13:30 h, and 13:30 h - 15:30 h. The amount of food consumed by fish was compared between these three time intervals at both 15 and 30 minutes of feeding. No significant differences existed between all three time intervals at either 15 or 30 minutes following food administration (Fig. 2-3).

2.3.1.4 Seasonal stage (gonadal phase) and food intake To determine if food intake varies in goldfish during the seasonal stages of gonadal late recrudescence (January - April), mature (post-spawning April - May), regressed (June - August), and early recrudescence (September - December), data were pooled and compared from unhandled male and female fish in each reproductive phase. At both 15 minutes (Fig. 2-4 a) and 30 minutes (Fig. 2-4 b) of feeding, gonadal regressed fish consumed a significantly greater amount of pellets relative to fish in late recrudescent and mature seasonal stages. Early recrudescent fish fed at levels which were not different from those recorded during all other seasonal stages.

2.3.1.5 Food intake in male and female goldfish Feeding data were pooled and compared from unhandled male or female fish in either gonadal late recrudescent or mature stages. No significant differences existed between females and males of the two gonadal sexual phases at both 15 and 30 minutes of feeding (Fig. 2-5 a and b). Additionally, no differences existed in the feeding levels between gonadal late recrudescent and mature males, or between females of both gonadal stages (Fig. 2-6).

2.3.2 Feeding and Serum GH Levels in Goldfish

2.3.2.1 Postprandial serum GH levels in goldfish Fish were anesthetized, weighed, and randomly sorted into 8 tanks which received aerated water at 17 - 19^{o}C and were

exposed to Edmonton natural photoperiod. Fish were fed once daily a prescibed ration of floating pellets at 09:30 h for approximately 10 days prior to experimentation. On experimental day, 4 groups of fish were fed a 2% bw ration while 4 groups remained unfed. At 15, 30, 60, and 240 minutes following food administration, one group of fed and one group of unfed fish were anesthetized and sampled for blood.

Circulating serum GH levels in goldfish were significantly increased at 30 minutes following feeding (Fig. 2-7). Hormone levels subsequently declined relative to starved controls at 60 minutes, and levels remained lower than in unfed fish up to four hours following food intake.

2.3.2.2 Serum GH levels in large and small goldfish at 30 minutes post-feeding Fish of either mean weight of 47 g (large) or 19 g (small) were randomly sorted into 8 tanks (n = 6 fish/tank; four tanks large fish, four tanks small fish) and acclimated under feeding and photoperiod conditions similar to previous experiments. On experimental day, two groups of large fish and two groups of small fish were fed a 2% bw ration. At 30 minutes following feeding, groups of fed and their unfed counterparts were anesthetized and sampled for blood.

Relative to unfed fish, both large and small fish exhibited significantly elevated serum GH levels following feeding (Fig. 2-8).

2.3.2.3 Serum GH levels in goldfish at 1.5 hours post-feeding of varied ration sizes Fish were anesthetized, weighed, and randomly sorted into 4 tanks and acclimated under feeding and photoperiod conditions similar to previous experiments. On experimental day, groups of fish received either a 1%, 2%, or 3% bw ration of pellets or remained unfed. At 1.5 hours post-feeding, all groups of fish were anesthetized and sampled for blood.

Relative to unfed controls, fish fed either a 2% or 3% bw ration exhibited a significant decrease in serum GH levels (Fig. 2-9).

2.4 Discussion

2.4.1 Goldfish Feeding Behavior

Initial studies in this chapter were conducted to develop a standard protocol whereby food intake could be accurately measured in goldfish. Based on findings from these experiments, a feeding behavior protocol was designed which was subsequently employed in feeding experiments involving the investigation into the effects of neuropeptides on food intake in goldfish (Chapters 3 and 6). Many physical and mechanical factors influence the amount of food consumed by fish including dietary composition, feeding frequency, gastric distention and feed evacuation rates, starvation period, ambient temperature and photoperiod (for reviews Peter, 1979; Holmgren *et al.*, 1983; Weatherley and Gill, 1987). However, using this established protocol many variables could be standardized and as such, variations in the basal levels of feeding between individual fish could be minimized.

Using the developed feeding protocol, goldfish were acclimated in aquaria containing warm water temperature and aquatic vegetation. In other experiments this environment has been shown to be suitable for reproductive and spawning behavior in goldfish. Stacey *et al.* (1979) and Stacey (1987) reported that under similar holding conditions, goldfish will undergo spawning behavior in the early morning, with the aquatic weeds, long light cycle, and warm water providing stimulatory cues to trigger this event. Data from experiments in 2.3.1 indicate that under these same holding conditions and through the use of specific floating fish pellets, the amount of food consumed by individual fish can be accurately measured. Data presented in this chapter on feeding behavior are in agreement with other limited, earlier feeding studies conducted in goldfish (Rozin and Mayer, 1961, 1964).

Fish in the standardized feeding protocol were exposed to a fixed ration of floating pellets for one hour during a 24 h period. Deprivation of food for 23 h assured that gut emptying was complete from the previous meal (Rozin and Mayer, 1964), and that appetite was comparable among individual fish before the start of an experiment. Under these standard conditions goldfish consumed approximately 1.1% body weight per hour. Even in the presence of handling and saline injection, this basal level of feeding remained unchanged over seven days (Fig. 2-2 a). In line with these findings are earlier studies in goldfish which demonstrate that the amount of food consumed by fish remains fairly

uniform over several days when fish are exposed to a similar feeding schedule (1h food intake and 23 h food deprivation) (Rozin and Mayer, 1964). Additionally, goldfish which are accustomed to receiving food for only one hour daily, will ingest the same quantity of food as goldfish deprived of food from 4 to 47 h; this is likely due to the absence of a stomach and a limited capacity to store food (Rozin and Mayer, 1964).

In 2.3.1.1 and 2.3.1.2, anesthetization, handling, and ip saline injection did not significantly alter cumulative food intake levels when compared to unhandled fish at 15, 30, 45, and 60 minutes during feeding (Fig. 2-1 c), indicating that such experimental manipulation results in limited stress. Following handling and injection, goldfish still exhibited the characteristic feeding behavior traits of fish such as: (1) arousal or awareness of the presence of food, (2) location and identification of the food, (3) intake to the mouth, (4) acceptance of the food, and ingestion into the gastrointestinal tract (for review Cowey *et al.*, 1985). These goldfish also exhibited the characteristic "area copying" behavior, where the location of food pellets by one fish immediately notified other fish in the tank of the food source present (Magurran, 1984; Pitcher 1986). Together these data indicate that goldfish are an excellent experimental model which can be manipulated and the acute effects on feeding responses measured.

The significant elevation in cumulative food intake witnessed during the gonadal regressed stage in goldfish (Fig. 2-4 a and b) indicates that data collected during this phase of reproduction must be treated in isolation from feeding data obtained in other gonadal reproductive stages. It is speculated that this increase in feeding during gonadal regression reflects a period when less body energy stores are required for maturation of the gonads and as such, energy is shunted into feeding and growth. In support of this, Marchant and Peter (1986) and Marchant *et al.* (1986) reported that goldfish, which are fed twice daily and are exposed to Edmonton natural photoperiod and ambient temperatures, exhibit the fastest growth rates in July, a period when fish are in gonadal regression.

When cumulative food intake was examined over the light phase, no significant differences in feeding levels were present when data were pooled into three time intervals (09:30 - 11:30 h; 11:30 - 13:30 h; 13:30 - 15:30 h). These data indicated that food intake measurements in individual fish could be conducted between 09:30 - 15:30 h and then

results could be pooled for an overall response. These findings also support other limited studies by Rozin and Mayer (1964) who reported that conditioned goldfish will distribute their feeding responses fairly evenly over the light cycle.

Finally, data in this chapter revealed that cumulative food intake levels remain similar between males and females in either late recrudescent or mature seasonal stages of gonadal reproduction, indicating that either sex may be used in experiments related to feeding behavior (Fig. 2-5 a and b).

2.4.2 Feeding and Serum GH Levels in Goldfish

Experiments in this chapter (2.3.2) demonstrate that associated with food intake in goldfish are rapid changes in circulating serum GH levels. Fish fed a 2% bw ration exhibit an acute increase in serum GH levels within 30 minutes of food administration (Fig. 2-7). This initial postprandial rise in serum GH was present in fish with a mean weight of either 19 g or 40 g (Fig. 2-8). In a second phase, serum GH levels of fed fish decrease within 45 minutes (data not shown) to 60 minutes relative to postprandial GH levels at 30 minutes (Fig. 2-7). This feeding-mediated decrease in serum GH levels appears to be dependent on meal size; fish receiving either a 2% or 3% bw ration exhibit significantly lower GH levels relative to unfed controls or fish consuming only a 1% bw ration (Fig. 2-9). Furthermore, these changes appear specific for serum GH, since another circulating pituitary hormone, gonadotropin (GtH-II) was in most cases, unaltered following feeding (data not shown). It remains unknown by what mechanism food intake elevates serum GH levels, although it is likely that both neuronal and humoral events as a result of the sight, taste, and the ingestion of food participate in modifying serum GH. One likely possibility is that specific neuropeptides/neurotransmitters which are released during food intake act to modify GH. Such neural signals may act indirectly or directly at the level of the pituitary to release GH.

2.4.2 Summary

Overall, experiments in this chapter formed the basis of a feeding protocol employed in experiments of subsequent chapters which examined the effects of the two neuropeptides, bombesin and cholecystokinin, on feeding behavior in goldfish. Goldfish accustomed to a 2% bw ration and held at an ambient temperature of 17 - 19^{o}C consumed

1.1% bw ration per hour. This feeding behavior does not change significantly over the course of the light phase or between females and males in late recrudescent or mature gonadal seasonal stages, although in regressed goldfish, the amount of food consumed is significantly elevated relative to fish in other seasonal gonadal phases of the reproductive cycle.

Accompanying food intake are postprandial changes in circulating levels of GH, which may be the result of humoral and neural substances that are released during feeding. Initially, serum GH levels increase within 30 minutes of feeding, this increase does not appear to depend on the size of fish. In a second phase, serum GH levels are significantly decreased within 90 minutes of feeding; this suppression is dependent on the amount of food consumed. GH levels in fed fish remain low to at least 4 hours post-feeding.

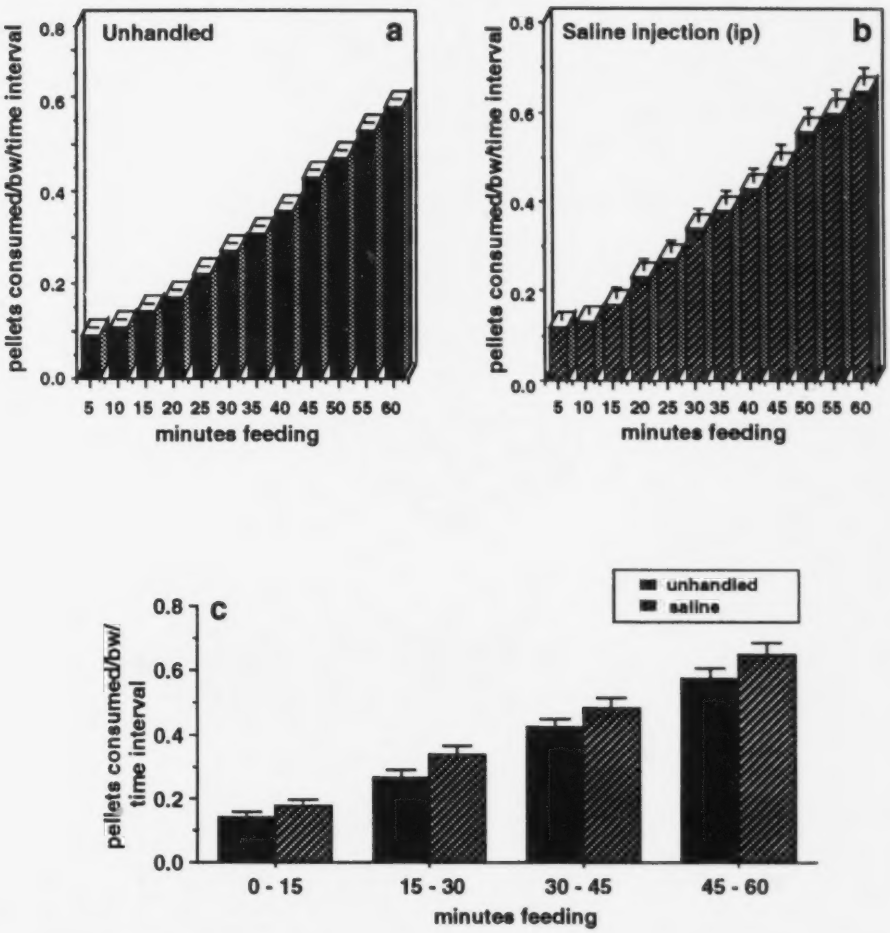

Fig. 2-1 (a, b, and c). Cumulative food intake in unhandled (a) and saline -injected (b) goldfish. On experimental day 1, the number of pellets consumed by unhandled fish was recorded over 60 minutes (a) while on experimental day 2, fish received an ip injection of saline 15 minutes prior to food administration. Food intake was then recorded for 60 minutes. No significant differences existed in the amount of food consumed between unhandled and saline-injected fish (c) (unhandled, n = 15; saline injected, n = 17).

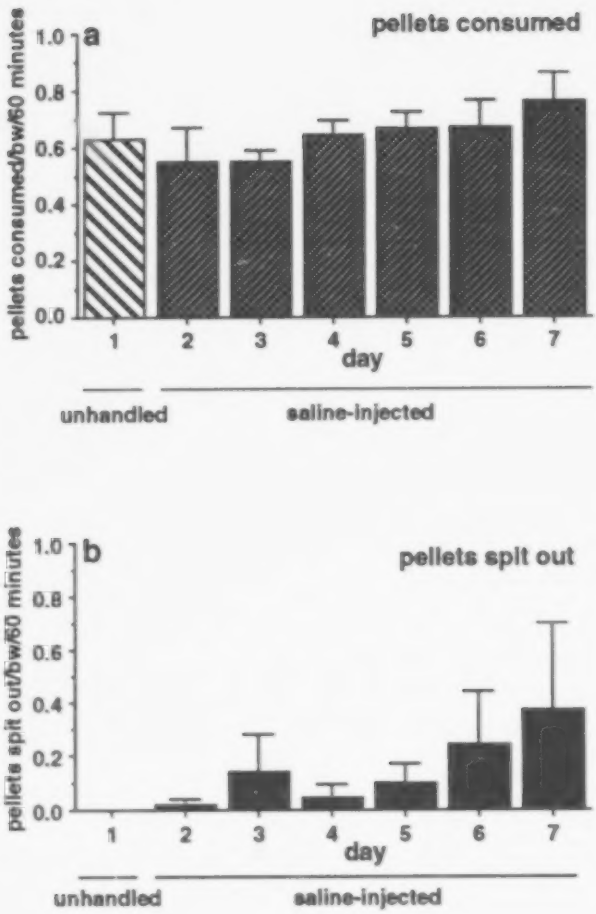

Fig. 2-2 (a and b). Effects of repeated saline injections on food consumption (a) and pellets spit out (b) in goldfish. On experimental day 1, food intake was measured in unhandled fish for 60 minutes. On days 2 - 7, fish were anesthetized and ip injected with saline at 15 minutes prior to food administration and measurement of food intake (n = 6 fish).

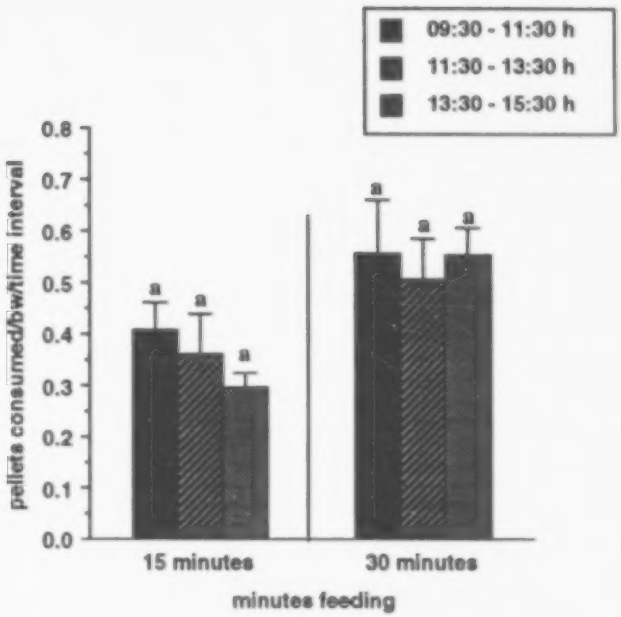

Fig. 2-3. Cumulative food intake in goldfish over the light cycle. Cumulative food intake was recorded in unhandled fish which had been acclimated to a 2% ration of floating pellets and had been fed once daily at a prescribed time during the light phase (n = 8 fish/group).

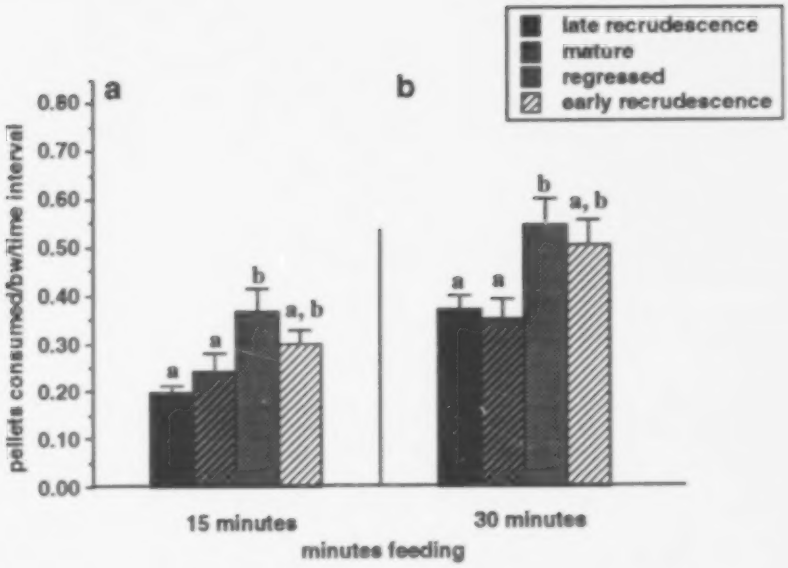

Fig. 2-4 (a and b). Seasonal changes in cumulative food intake in goldfish. Food intake was measured in unhandled goldfish during 15 minutes (a) and 30 minutes (b) of feeding while in the seasonal gonadal stages of late recrudescence, regressed, mature, and early recrudescence (n = 12-33 fish/group).

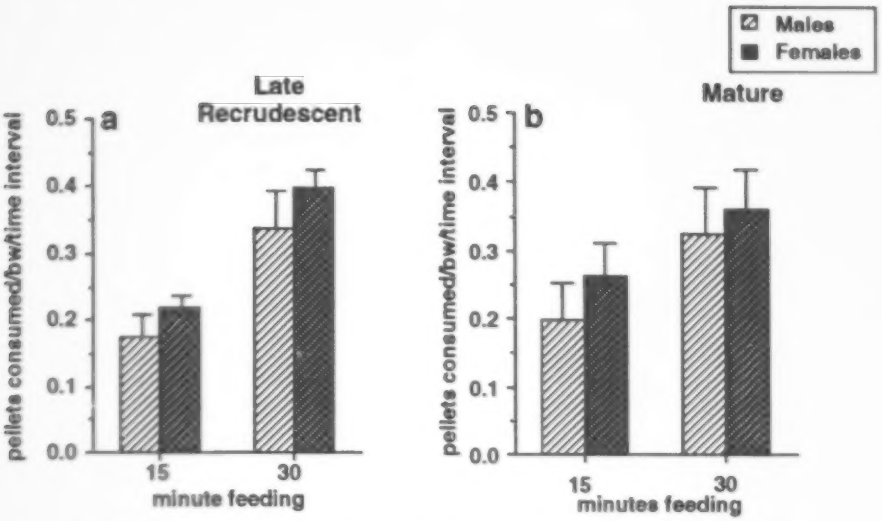

Fig. 2-5 (a and b). Food intake in male and female goldfish. The amount of food consumed by fish in either recrudescent or mature gonadal stages was recorded during 30 minutes of feeding (n = 9-19 fish/group).

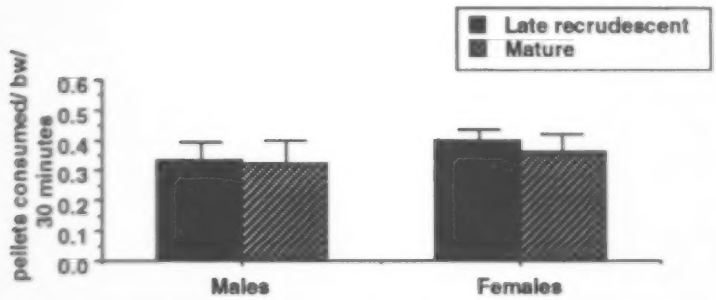

Fig. 2-6. Food intake in goldfish of late recrudescent and mature gonadal stages. Male and female fish in late recrudescent and mature gonadal stages exhibited no significant differences in cumulative food intake (n = 9 - 19 fish /group)

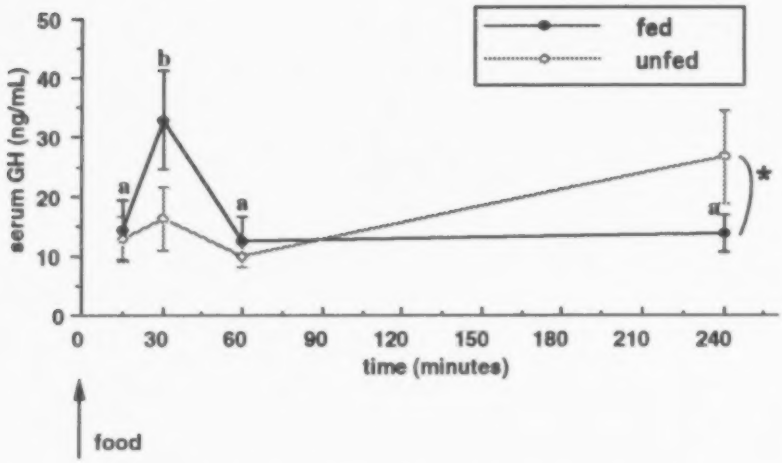

Fig. 2-7. Postprandial serum GH profile in goldfish. Fish were either fed a 2% ration or remained unfed. At 15, 30, 60, and 240 minutes following food adminstration, one group of fed and one group of unfed fish were sampled (n = 8 fish/group). Asterisk represents significant difference between groups with Student's t-test, $p < 0.05$.

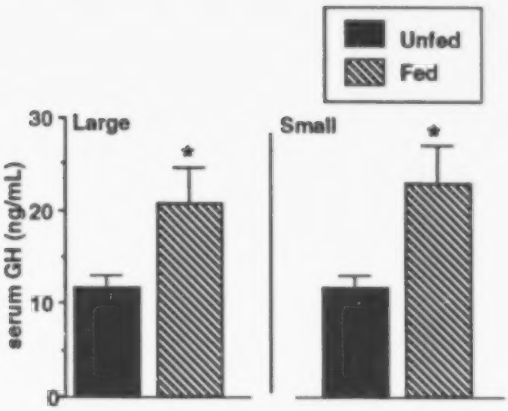

Fig. 2-8. Feeding and serum GH levels in large (40 g) and small (19 g) goldfish. Fish were either fed a 2% ration or remained unfed (controls). Fish were sampled at 30 minutes following feeding (n = 12 fish /group).

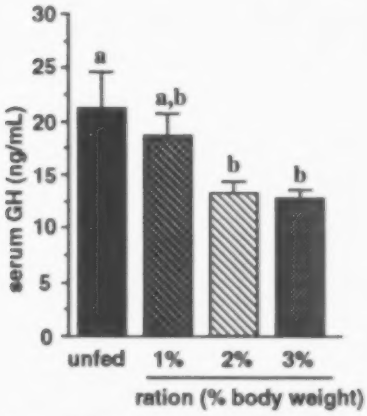

Fig. 2-9. Effects of varied ration size on serum GH levels in goldfish. Fish were fed either a 1%, 2%, or 3% ration or remained unfed (controls). At 1.5 hours post-feeding, all groups of fish were sampled (n = 12 fish/group).

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Chapter 3

Bombesin Acts to Suppress Feeding Behavior and Alter Serum Growth Hormone In Goldfish[1]

3.1 Introduction

The tetradecapeptide bombesin (BBS) was first isolated from skin extracts of the fire bellied toad, *Bombina bombina* (Anastasi *et al.*, 1971), but has since been shown to belong to a family of BBS-like peptides which are present throughout all vertebrate classes (refer to Chapter 1). In mammals, both BBS and gastrin releasing peptide (GRP), a 27 amino acid peptide which shares a similar decapeptide C-terminal sequence as BBS, have been shown to be widely distributed within nerves of the central nervous system and gastrointestinal tract, where they regulate feeding-related activities (for review McCoy and Avery, 1991). When administered either intraperitoneally (ip) or centrally within specific areas of the hypothalamus and/or the hindbrain, BBS-related peptides are potent in suppressing food intake (Gibbs *et al.*, 1979; Stuckey and Gibbs, 1982; Ladenheim and Ritter, 1989; Flynn 1991, refer to Chapter 1). In addition, BBS-related peptides have been shown to regulate other feeding related processes, such as gastrin and gastric acid secretion, exocrine and endocrine pancreatic secretion, and gastrointestinal motility (Vaysse *et al.*, 1981; Hostetler *et al.*, 1989).

BBS/GRP-like peptides have also been localized within the mammalian anterior pituitary where they may act to regulate hormone release. Steel *et al.* (1992) reported BBS-immunoreactive (IR) cells within the anterior pituitary of several mammalian species, while somatotropes, corticotropes, and lactotropes of the rat pitutiary have been shown to contain GRP-IR material (Houben and Denef, 1991). Pro-GRP-IR has also been described within rat anterior pituitary cells, suggesting local synthesis of BBS/GRP-like peptides (Houben and Denef, 1991). Within the anterior pituitary, BBS/GRP-related peptides may play a physiological role in regulating growth hormone (GH) release (Rivier *et al.*, 1978; Westendorf and Schonbrunn, 1982; Bicknell and

[1]A version of this chapter has been published: Himick BA and Peter RE 1994. Physiology and Behavior. 55: 65-72.

Chapman, 1983; Houben, Denef and Vranckx, 1990), subsequently causing an increase in circulating GH levels (Kentroti *et al.*, 1988). BBS has also been shown to interact with somatostatin and GH-releasing hormone to stimulate circulating serum GH (Kentroti and McCann, 1985; Kentroti *et al.*, 1988).

In fish, BBS/GRP-like peptides have been detected within neurons and endocrine cells of the gastrointestinal tract (for review Bjenning and Holmgren, 1988; Holmgren and Jonsson, 1988; Bjenning *et al.*, 1990), in the cardiovascular system (Bjenning *et al.*, 1990) and more recently, in the brain (Vallarino *et al.*, 1990). BBS/GRP-like peptides have also been isolated from the intestine of the elasmobranch, *Scyliorhinus canicula* (Conlon *et al.*, 1987), and the stomach of the rainbow trout, *Oncorhynchus mykiss* (Jensen and Conlon, 1992). In fish, BBS-like peptides are active peripherally in regulating gut motility and visceral activity (Holmgren *et al.*, 1982; Thorndyke and Falkmer, 1982; Holmgren and Jonsson, 1988; Thorndyke and Holmgren, 1990; Bjenning *et al.*, 1991), but the actions of BBS-like peptides within the teleost central nervous system remain unknown.

Despite the presence of BBS/GRP-like peptides within the teleost gut and the apparent involvement of BBS-like peptides in the regulation of teleost gut function, there exists no detailed information on the effects of BBS, or any neuropeptide for that matter, on the peripheral and central regulation of feeding behavior, with the exception of one preliminary study demonstrating a decrease in food consumption by carp following peripheral BBS injection (Beach *et al.*, 1988). Therefore, studies in this chaper have investigated the actions of BBS on feeding behavior in the goldfish Since pituitary GH may also play a role in the regulation of appetite of fish (refer to Chapter 1), serum GH levels were also examined in BBS-injected fish.

Changes in circulating serum GH levels which accompany BBS-induced feeding suppression in the goldfish suggest that a relationship may exist between BBS and GH during altered feeding activity. Overall, these studies are unique in that they represent the first detailed report, in any lower vertebrate, of a neuropeptide which is capable of altering feeding behavior when administered either peripherally or into the brain ventricular system.

3.2 Materials and Methods

Experimental Animals

Male and female goldfish (25-45 g) of the common or comet varieties, were purchased from Ozark Fisheries (Stoutland, MO). Fish were maintained under simulated Edmonton natural photoperiod in 1.8 kL tanks which received a constant flow of aerated and dechlorinated city water at 17°C. Fish were fed *ad libitum* twice daily with commercially prepared fish pellets.

Observational Experiments

To examine the effects of BBS on feeding behavior, fish were anesthetized with 0.05% tricaine methanesulfonate (TMS: Syndel laboratories, Vancouver, BC), weighed to the nearest 0.1 g and randomly assigned to 65 L observational aquaria containing flow-through, aerated water at 17 - 19°C (3 fish per aquaria: photoperiod 16L : 8D). Fish were identified by individual markings. All test aquaria contained gravel substrate and floating synthetic weeds, while tank sides were covered by opaque barriers to minimize external disturbances. Following the protocol developed in Chapter 2, goldfish were fed once daily a 2% bw ration of floating pellets (3PT Fish Food Float, Martin Feed Mills Ltd., Elmira, Ontario). Observational experiments were conducted under standardized conditions in female and male fish which were in the seasonal stages of gonadal regressed or recrudescence (refer to Chapter 2). On experimental day, fish were anesthetized and injected ip (3 µL/g) or into the third brain ventricle with either saline or peptide. Fish were replaced back into their respective tanks and allowed to recover. To record the amount of food consumed per fish on experimental day, a 2% bw ration of floating pellets was added at 15 minutes post-injection and each pellet consumed by individual fish within the tank was recorded during 30 minutes. Over the 30 minute feeding period, size of ration was not found to be limiting. Measurement of food intake began immediately upon entry of pellets into the tank. Food consumption was converted to milligrams of food consumed/wet body weight/15 or 30 minutes feeding based on the mean pellet weight fed to fish.

In all experiments except (3.3.1.2), blood samples were collected immediately following the observation period. Fish were netted and anesthetized and, when fin and

body movements ceased, a 500 µL blood sample was collected from the caudal vasculature using a 25-gauge, 5/8 inch needle attached to a 1 mL syringe. After clotting for approximately 3-4 h at 4°C, samples were centrifuged and serum was collected and stored at -28°C until GH hormone analyses were conducted.

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Ventricular Injection into the Goldfish Brain

Ventricular brain injections (icv) were administered following routine procedures outlined in the stereotaxic atlas for the goldfish forebrain (Peter and Gill, 1975). Briefly, a dental blade was used to cut three sides of a bone flap in the roof of the skull. The bone flap was then folded back, the brain was exposed, and the injection needle was placed stereotaxically in the brain ventricle. Following injection (2 µl) of either goldfish physiological saline or peptide, the needle was withdrawn and the skull flap was replaced. Fish were returned to their tanks and normally recovered from anesthesia within two to three minutes. Icv injections were administered at brain co-ordinates of +1.3 mm (anterior to posterior commissure), midline at a depth of +1.8 mm, giving an approximate placement in the brain ventricle in the region of the nucleus preopticus and nucleus preopticus periventricularis (Peter and Gill, 1975).

Peptides Administered

Synthetic bombesin -14 and neuromedin C were purchased from Bachem Bioscience Inc. (Philiadelphia, PA).

Hormone Measurement and Data Analysis

Concentrations of serum GH levels were determined by a specific radioimmunoassay (RIA) using purified common carp GH as standards and as radioligands. A description of the carp GH RIA has been documented elsewhere (Marchant *et al.*, 1989; refer to Chapter 2 for details of RIA procedure). Briefly, the RIA was performed using a double antibody method; serum samples were incubated in a 0.08M sodium barbital buffer containing 2.5% normal rabbit serum and rabbit anti-carp GH. After 24 h incubation at 4°C, ^{125}I-carp GH was added to each tube and incubation was continued for an additional 24 h at 4°C. Precipitation of the antibody-bound hormone was by addition of diluted goat ant-rabbit gamma-globulin.

Differences in serum GH levels between groups of fish were analysed using one way ANOVA, followed by Duncan's multiple range test. For observational experiments, differences in the amount of food consumed between control and experimental fish were analysed using either Student's t-test or ANOVA, followed by Duncan's multiple range test. For all experiments, significance was considered at $p \leq 0.05$.

3.3 Experiments and Results

3.3.1 Effects of BBS on Feeding Behavior and Serum GH Levels

3.3.1.1 Dose response of intraperitoneal BBS injection on food intake To examine the effects of ip administration of BBS on food intake, fish which had received food 24 h earlier, were anesthetized and injected with either saline (0.9% NaCl) or one dose of BBS (0.5 - 100 ng/g). Food intake was recorded at 15 minutes post-injection.

Basal feeding levels were recorded in unhandled fish on day 1 (Fig. 3-1 a) to compare the amount of food consumed by these same fish when netted, anesthetized, and saline injected on experimental day 2 (Fig. 3-1 b and c). Data presented here, as well in Chapter 2, indicate that experimental manipulation of goldfish involving handling and ip injection prior to feeding, does not alter the subsequent cumulative feeding frequency in fish during 30 minutes of food intake when compared to levels observed in an unhandled state.

Five sequential experiments were conducted to examine the effects of varying doses of BBS on food intake in goldfish. Since the mean amount of food consumed by saline-injected fish did not significantly differ between the five experiments, data were pooled to construct an overall dose-response effect of BBS on feeding behavior (Fig. 3-1 b and c). At 15 minutes following feeding, 50 ng/g BBS caused a 91% suppression in food intake, whereas at 30 minutes following feeding 24 and 50 ng/g BBS decreased food intake to 68% and 88% of controls, respectively. At 45 minutes post-injection, serum GH levels were elevated in fish receiving 50 ng/g BBS relative to saline -injected controls (Fig. 3-1 d).

3.3.1.2 Effects of intraperitoneal BBS injection of food intake and food expelled Feeding behavior was recorded in unhandled fish on day 1. On day 2 these same fish were injected with saline and cumulative food intake was recorded. On day 3, the same fish were injected with 50 ng/g BBS and the cumulative number of pellets either consumed or taken into the buccal cavity but spit out, were recorded for 30 minutes. While the amount of food consumed did not vary between unhandled fish on day 1 and saline-injected fish on day 2, feeding behavior was suppressed in BBS-injected goldfish on day 3 over 15 minutes and at the end of 30 minutes of feeding (Fig. 3-2 a and b). Although fish consumed fewer pellets following BBS injection, they still searched for food and took pellets into their buccal cavity, but expelled or "spit out" the food at a greater frequency relative to the two previous feeding intervals (Fig. 3-2 a and b). This "spitting out" behavior was observed in fish injected with doses as low as 5 ng/g BBS (data not shown).

3.3.1.3 Effectiveness of intraperitoneally injected BBS-mediated feeding suppression Fish were unfed for 72 h, and then ip injected with 50 ng/g BBS. Cumulative food intake was recorded for 30 minutes. Relative to saline-injected controls, BBS-injected goldfish exhibited a 87% and 80% suppression in food intake at 15 and 30 minutes of feeding, respectively (Fig. 3-3 a and b). Associated with a decrease in food consumption in BBS-injected fish was a significant elevation in serum GH levels (Fig. 3-3 c).

3.3.1.4 Duration of intraperitoneally injected BBS-mediated feeding suppression Fish were ip injected with 50 ng/g BBS and at 3 h post-injection cumulative food intake was recorded for 30 minutes. BBS-injected goldfish exhibited a 56% suppression in food intake at the end of 30 minutes of feeding, relative to saline-injected controls (Fig. 3-4).

3.3.1.5 Effects of third brain ventricle injection of BBS on feeding behavior and serum GH Fish were anesthetized and injected icv with 2 μL of either goldfish physiological saline or 60 ng/g BBS. Fifteen minutes post-icv injection, fish were administered their 2% bw ration and cumulative food intake was recorded for 30 minutes. On the day prior to experimentation, basal feeding levels were recorded in these same fish to compare the effects of handling and icv injection on feeding behavior relative to fish in an unhandled

state. Comparison of cumulative feeding levels in unhandled fish on day 1 (Fig. 3-5 a) and fish injected with saline on day 2 (Fig. 3-5 b) indicate that experimental manipulation involving netting, anesthetization, and icv injection prior to food presentation does not alter cumulative feeding frequency over 30 minutes of feeding. Central administration of BBS into the third brain ventricle significantly suppressed cumulative food intake after 15 minutes (78%) and at the end of 30 minutes (82%) of being presented with food (Fig. 3-5 b). Accompanying suppressed feeding behavior in BBS-injected fish was a significant two fold elevation in serum GH levels at 30 minutes following feeding (Fig. 3-5 c). Unlike the effects observed with peripherally administered BBS, fish exhibited no behaviors associated with expelling or "spitting out" of pellets following central injection of BBS. Suppressed feeding behavior was also observed in fish which were injected icv with a dose of 10 ng/g BBS (data not shown).

3.3.1.6 Effects of neuromedin C (NMC) on feeding behavior and serum GH Fish were ip injected with equimolar doses of BBS or NMC (0.01 mM; 3 μL), and at 15 minutes post-injection cumulative food intake was recorded for 30 minutes. BBS-injected goldfish exhibited a 69% suppression in food intake at the end of 30 minutes of feeding, while NMC injection resulted in only a 40% decrease in food intake, relative to saline-injected controls (Fig. 3-6a). No changes in circulating serum GH levels were observed in fish which were injected with either BBS or NMC.

3.3.2 Effects of BBS on Circulating Serum GH Levels

3.3.2.1 Dose response of intraperitoneal BBS injection on serum GH Prior to experimentation, fish were anesthetized, weighed to the nearest 0.1 g, and randomly sorted into 4 tanks which received aerated water at 19^{o}C and were exposed to Edmonton natural photoperiod. Fish were fed once daily their prescribed ration of food at 09:30 h for approximately 10 days. On the experimental day, groups of fish were anesthetized and injected with either saline or one dose of BBS (1.0, 10.0, or 100 ng/g). Fish were replaced in their respective tanks and allowed to recover. At 1.5 h post-injection, all 4 groups of fish were anesthetized, sampled for blood, and serum collected.

Groups of fish receiving an ip injection of 10 and 100 ng/g BBS exhibited significant

increases in serum GH levels at 1.5 h post-injection, relative to saline-injected controls (Fig. 3-7).

3.3.2.2 Dose response of third brain ventricle BBS injection on serum GH Prior to experimentation, fish were randomly sorted into 6 tanks (n = 6 fish/tank) which received aerated water at 19^{o}C and were exposed to Edmonton natural photoperiod. On the experimental day, fish were anesthetized and two groups were injected with saline, while two groups were injected with either 5 or 50 ng/g BBS into the third brain ventricle at similar co-ordinates as those used in (3.3.1.5). Injections took place over a staggered sequence to permit adequate time for sampling. At 1.5 h post-injection, groups of fish were anesthetized, sampled for blood, and serum collected. Third brain ventricle injection of 5 or 50 ng/g BBS resulted in a significant increase in serum GH levels at 1.5 h post-injection, relative to saline-injected controls (Fig. 3-8).

3.3.2.3 Effects of BBS on Serum GH Levels in the Presence and Absence of Food Fish were randomly sorted into 4 tanks and, on experimental day, fish in two groups received an injection of saline while fish in the remaining two groups received an injection of 100 ng/g BBS. At 15 minutes post-injection, one saline-injected group and one BBS-injected group were fed a 2% bw ration, while the other two groups remained unfed. At 1.5 h post-injection, blood samples were collected from all 4 groups of fish. Fish receiving an ip injection of BBS in the presence or absence of food exhibited significantly elevated serum GH levels relative to groups of fish receiving saline with or without food (Fig. 3-9). No differences existed in GH levels between the two groups of BBS-injected fish, indicating that serum GH levels are altered by BBS regardless of the presence or absence of food.

3.3.2.4 Effects of BBS on Postprandial Serum GH Profile Fish were randomly sorted into 8 tanks and on experimental day, 4 groups of fish were injected at time zero (T = 0) with saline and 4 groups were injected with 50 ng/g BBS. Fifteen minutes post-injection and before food entry into tanks, one group of saline and one group of BBS -injected fish were anesthetized and sampled for blood. The remaining 6 tanks received food in excess (5% bw ration) at 15 minutes post-injection, and one group of fed and one group of

unfed fish were sampled for blood at 30, 60, and 120 minutes post-injection. In groups of fed, saline-injected controls, serum GH levels were increased at 15 minutes following feeding after which levels were significantly depressed at 60 and 120 minutes following feeding. On the contrary, fish which received an ip injection of 50 ng/g BBS just prior to food presentation exhibited significantly lower serum GH levels at 30 minutes post-injection when compared to saline-injected controls (Fig. 3-10).

3.4 Discussion

3.4.1 BBS and Feeding Behavior

Data in this chapter demonstrate that BBS acts acutely to suppress feeding behavior in the goldfish when administered either peripherally, or centrally within the third brain ventricle. When injected ip into fish which had been fed 24 h previously, BBS induced a dose-dependent suppression in food consumption over a dose range of 0.5 ng/g to 50 ng/g. At 30 minutes post- BBS injection (15 minutes following presentation of food), 50 ng/g BBS induced a 91% decrease in the mean amount of food consumed, whereas at 45 minutes following BBS injection, 24 ng/g and 50 ng/g BBS significantly inhibited food intake. BBS (50 ng/g) was still as effective in suppressing food intake within fifteen minutes in goldfish which had been deprived of food for 72 h, indicating the effectiveness of this neuropeptide in mediating effects on feeding behavior. NMC (GRP10) also decreased food intake, but was not as effective as BBS when administered at an equimolar dose. It has been shown that NMC binds with high affinity to the BBS receptor in goldfish (Chapter 7); these data demonstrate, however, that BBS is more efficacious in suppressing feeding when peripherally injected. These results parallel similar findings documented in higher vertebrates, including the rat, where earlier studies by Gibb *et al.* (1979) described suppressed feeding within 15 minutes of an ip injection of 2 ng/g to 16 ng/g BBS. More recently, McCoy *et al.* (1990) reported BBS-induced feeding suppression in the rat based on dietary selectivity; ip injection of BBS at doses similar to the range of doses used in these studies resulted in a significant reduction in predominantly carbohydrate and protein intake within 30 to 60 minutes of administration.

Associated with ip BBS injection and feeding suppression in goldfish was a second behavior which involved "spitting out" or expelling of pellets from the buccal cavity.

This was observed frequently in other experiments not reported here, and may provide evidence of a mechanism whereby peripherally administered BBS mediates its suppressive effects on food consumption in fish. In other fish species, such as *G. morhua*, and *O. mykiss*, BBS-like peptides have been reported to stimulate the contractility of longitudinal and circular stomach muscles *in vitro* (Holmgren and Jonsson, 1988; Thorndyke and Holmgren, 1990). Furthermore, in the elasmobranch, *S. acanthias*, BBS has been reported to alter blood flow within the gastrointestinal vasculature indicating the involvement of BBS in the regulation of the gut circulatory system (Bjenning *et al.* 1990). In species more closely related to the goldfish, such as the carp (*Cyprinus carpio*), an abundance of BBS-IR material has been reported within the intestine and rectum (Bjenning and Holmgren, 1988). In the present experiments, where BBS was peripherally administered to goldfish, enhanced gastrointestinal contractility may have played a "mechanical" role in decreasing the amount of food consumed. Concomitantly, alterations in blood circulation within the gut following BBS administration may have contributed in mediating feeding signals via a humoral action.

BBS also acted to suppress feeding behavior in goldfish following injection into the third brain ventricle. When 60 ng/g BBS was icv injected, food consumption was decreased to 78% and 82% of control fish during 15 and 30 minutes of their feeding interval, respectively. To our knowledge, these findings are the first to demonstrate the suppressive actions of a neuropeptide on feeding behavior via the brain in any lower vertebrate, and are in accordance with other studies involving BBS/GRP-like peptides conducted in mammals (Stuckey and Gibbs, 1982; Willis *et al.*, 1984, for review Morley, 1987). Presently, it is not known where in the goldfish brain BBS acts to mediate it's suppressive effects on feeding behavior. Although BBS was injected in a region which was surrounded by the nucleus preopticus and nucleus preopticus periventricularis, access of the peptide to all sites in contact with the ventricular system and cerebrospinal fluid likely occurred making it impossible to ascertain a specific site of action. Based on earlier anatomical studies illustrating gustatory and medial forebrain bundle connections to the inferior lobe of the teleost hypothalamus (Herrick, 1905; Crosby and Showers, 1969; Finger, 1978), as well as electrical stimulation studies within specific brain areas of the goldfish and carp (Redgate, 1974; Savage and Roberts, 1975; Roberts and Savage, 1978), the bluegill sunfish (*Lepomis macrochirus*) and the

mouthbreeder, *Tilapia macrocephala* (Demski and Knigge, 1971; Demski, 1973; for review Demski, 1981; 1983; Chapter 2), areas within the inferior lobe of the hypothalamus, and in particular the nucleus recessus lateralis (NRL), appear to be involved in the regulation of feeding activity. The cell bodies of the NRL are exposed to the ventricular system, and may represent one likely site of action where BBS mediates its suppressive effects on feeding.

Recently, Kiliaan *et al.* (1992) failed to detect BBS-IR material in the gut of *C. auratus*. In addition, these authors observed no effects of BBS on electrogenic ion transport of the intestinal epithelia in goldfish. These findings are in discordance with findings of BBS-like IR in the goldfish in Chapter 4 and of other studies which show extensive BBS-IR within the nerves of the gastrointestinal tract of other closely related fish species within the Cyprinidae family (Bjenning and Holmgren, 1988). Evidence within several chapters of this thesis (Chapters 3, 4, 7) strongly indicate that BBS is present in the goldfish and that BBS-like peptides play a role in the regulation of feeding behavior and anterior pituitary hormone secretion in fish. In the goldfish, BBS-like peptides are capable of suppressing feeding behavior centrally and peripherally (Chapter 3), of directly releasing GH from pituitary fragments *in vitro* following perifusion of low doses (Chapter 4), and of altering circulating serum GH levels *in vivo* (Chapter 3). The presence of high-affinity, specific BBS/GRP-preferring receptors in the brain, pituitary, and gastrointestinal tract of the goldfish (Chapter 8) provide additional strong evidence that BBS-like peptides serve as physiological regulators of neuroendocrine and behavioral events in teleosts.

Several behavioral actions were displayed by fish in these experiments which indicate that BBS was likely not decreasing food intake through the production of malaise. Firstly, following BBS injection, the latency in which goldfish actively searched for food and accepted pellets remained unchanged relative to saline-injected controls. In other words, within 15 minutes of receiving pellets, BBS-injected fish were responsive to the food and exhibited only a suppression in the amount of pellets that they consumed. Secondly, feeding behavior actions displayed by BBS-injected fish, such as searching for food and acceptance of pellets into the buccal cavity, did not differ from those exhibited by saline-injected controls. Additionally, goldfish are known to be area copy feeders, where location of a food source by one fish serves to notify other fish of its presence and

as a result, group feeding occurs (Pitcher *et al.*, 1982). The fact that amongst BBS-injected fish were saline-injected controls which were eating at a frequency comparable to levels in unhandled fish, indicates the potency of BBS in its suppressive effects on feeding.

3.4.2 BBS and Serum GH Levels

Concomitant with decreased feeding following administration of BBS in goldfish were alterations in circulating serum GH levels, suggesting that BBS and GH may interact while food intake is being suppressed. Experiments in this chapter demonstrate that serum GH levels are elevated at 45 minutes to 90 minutes following ip injection of BBS at doses of 10 ng/g or greater. During this time interval however, the presence of food was not required for BBS to mediate an increase in serum GH levels. On the contrary, at 15 to 30 minutes post BBS-injection fish exhibited decreased serum GH levels relative to saline-injected fed controls. It appears that peripherally administered BBS may initially act to prevent the elevation in serum GH normally witnessed following a meal, but then subsequently mediate an increase in GH levels.

Data in this chapter reveal that feeding in either unhandled fish or fish which are anesthetized and then saline injected, exhibit a postprandial surge in serum GH levels within 15 to 30 minutes of food intake followed by a rapid decline in serum GH. Thus, the initial unchanged serum GH levels witnessed in the BBS-treated fish may actually represent circulating GH levels of an "unfed" fish. With little to no food intake, BBS-treated fish exhibit no surge in serum GH, and hence have lower initial serum GH levels relative to their fed counterparts. The later second phase, where BBS-injected fish exhibit an increase in serum GH levels may result from a direct action of BBS at the level ot the goldfish pituitary somatotope to release GH. Data in Chapter 4 indicate that BBS is capable of directly regulating the release of GH at the level of the goldfish pituitary. The anterior pituitary of the goldfish lies outside the blood-brain barrier (Kah *et al.*, 1983) and as such, peripherally administered BBS could have access to the somatotopes *in vivo* . It remains possible that this is one mechanism whereby alterations in circulating serum GH levels occurred following injection of BBS.

When BBS was injected into the third brain ventricle of the goldfish, a significant

increase in serum GH levels also resulted within 45 minutes of administration. Interestingly, these findings contradict mammalian studies, where icv injection of BBS/GRP-like peptides significantly inhibit circulating GH levels (Westendorf and Schonbrunn, 1982; Kabayama *et al.*, 1984; Kentroti and McCann, 1985). Here it is believed that BBS/GRP-like peptides interact with hypothalamic somatostatin (SRIF) to decrease the release of pituitary GH secretion (Yashuhiro *et al.*, 1984; Kentroti and McCann, 1985; Kentroti *et al.*, 1988). This effect is opposite to that observed in mammalian studies *in vitro*, where rat or bovine cultured pituitary cells release GH in response to BBS/GRP-like peptides (Rivier *et al.* 1978; Westendorf and Schonbrunn, 1982; Bicknell and Chapman, 1983; Kentroti and McCann, 1985; Houben *et al.*, 1990). In the present studies, it is unknown if icv injection of BBS altered serum GH levels via an interaction with a hypothalamic regulator of GH release, such as SRIF or a GH-releasing factor. Experiments involving icv injection of BBS were conducted in gonadal regressing goldfish, a seasonal period when SRIF-IR is at its lowest (Marchant *et al.*, 1989). Thus, if an interaction between BBS and SRIF on pituitary GH release does exist within the brain of the goldfish, the inhibitory effects of SRIF on pituitary GH secretion is at a minimum during this time. This may allow any stimulatory actions of BBS on pituitary GH secretion to prevail.

Overall, studies presented in this chapter demonstrate for the first time that BBS is capable of regulating feeding behavior in the goldfish by acting both peripherally or centrally within the brain. Associated with suppressed feeding following BBS administration are concomitant alterations in circulating serum GH levels, which may be the result of BBS acting directly at the level of the pituitary or through interaction(s) with hypothalamic regulators of pituitary GH secretion. Since circulating serum GH levels are altered through feeding alone in goldfish, and that BBS modifies this postprandial serum GH profile, it is possible that BBS and GH interact to regulate feeding behavior in the goldfish.

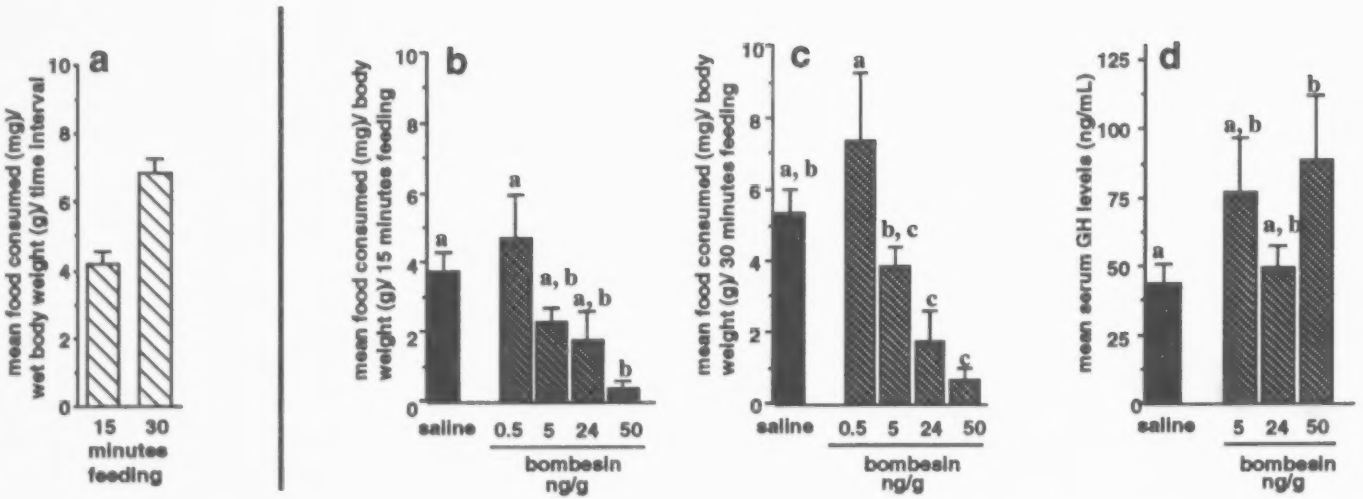

Fig. 3-1. Dose-dependent suppression of cumulative food intake in goldfish following intraperitoneal injection of increasing doses of bombesin. Cumulative food intake was recorded for 15 and 30 minutes in unhandled fish on day 1 (**a**). On day 2, fish were injected with either one dose of bombesin (0.5, 5.0, 24, or 50 ng/g) (hatched bars) or saline (solid bars) and, at 15 minutes post-injection, cumulative food intake was recorded for 15 and 30 minutes (**b** and **c**). Blood samples were collected immediately following 30 minutes of feeding observations for measurement of serum growth hormone levels (**d**). Since food intake in saline treated fish did not significantly differ between experiments, data was pooled to establish a dose-response effect of bombesin on feeding in goldfish (n = 7-9 fish/treatment, pooled saline controls n = 30).

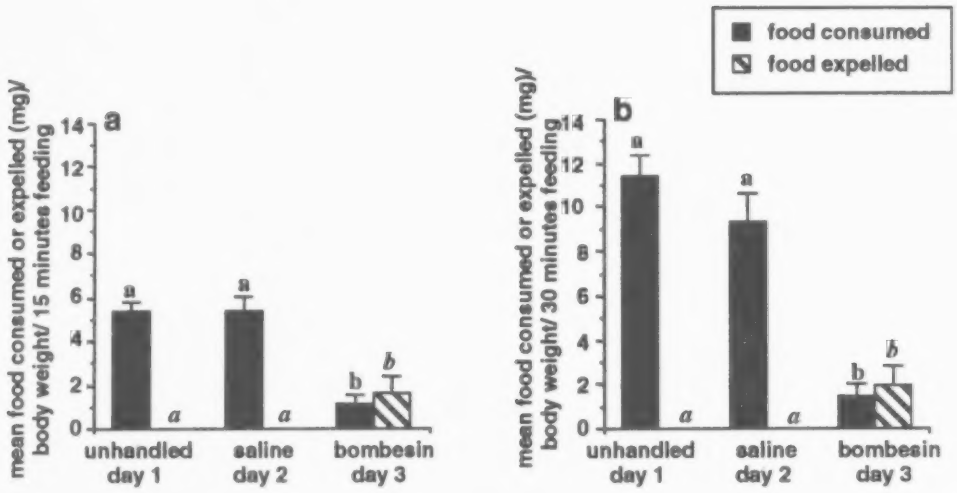

Fig. 3-2. Effects of intraperitoneal bombesin injection on cumulative food intake and food expelled in goldfish. Cumulative food intake (solid bars) and pellets expelled (hatched bars) were recorded for 15 (**a**) and 30 (**b**) minutes in unhandled fish on day 1, on day 2 at 15 minutes post-saline injection, and on day 3 at 15 minutes post-bombesin (50 ng/g) injection (n = 6 fish; lower case = difference between levels of food consumed; lower case italics = difference between levels of food expelled).

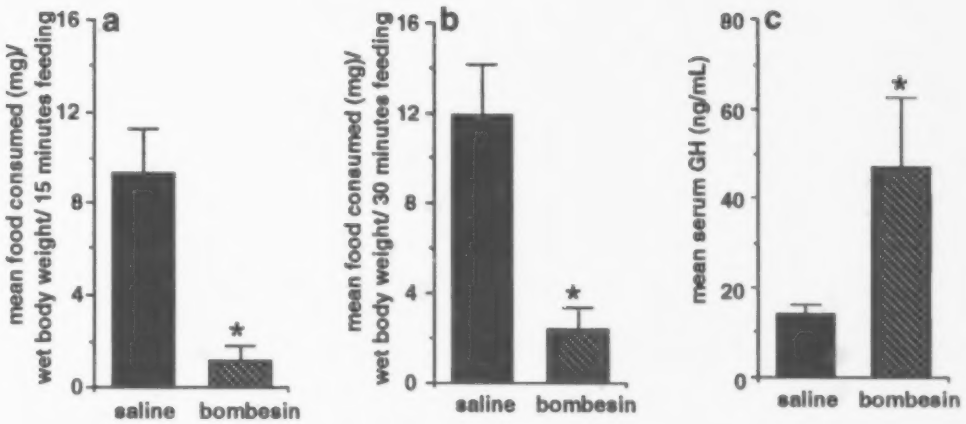

Fig. 3-3. Effectiveness of bombesin-mediated feeding suppression in goldfish. Bombesin (50 ng/g) was intraperitoneally injected into fish which had been deprived of food for 72 hours. At 15 minutes post-saline (solid bars) or bombesin (hatched bars) injection, cumulative food intake was recorded for 15 **(a)** and 30 **(b)** minutes. Serum growth hormone values **(c)** represent levels at the end of 30 minutes of behavioral observations (n = 9 fish/treatment).

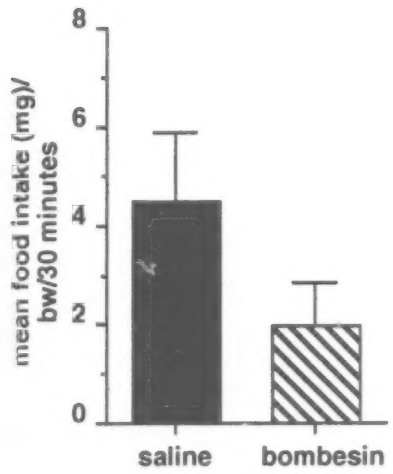

Fig. 3-4. Duration of intraperitoneally injected bombesin-mediated feeding suppression. Bombesin (50 ng/g) was intraperitoneally injected into fish and at 3 hours following peptide administration, cumulative food intake was recorded. (n = 7-8 fish/treatment).

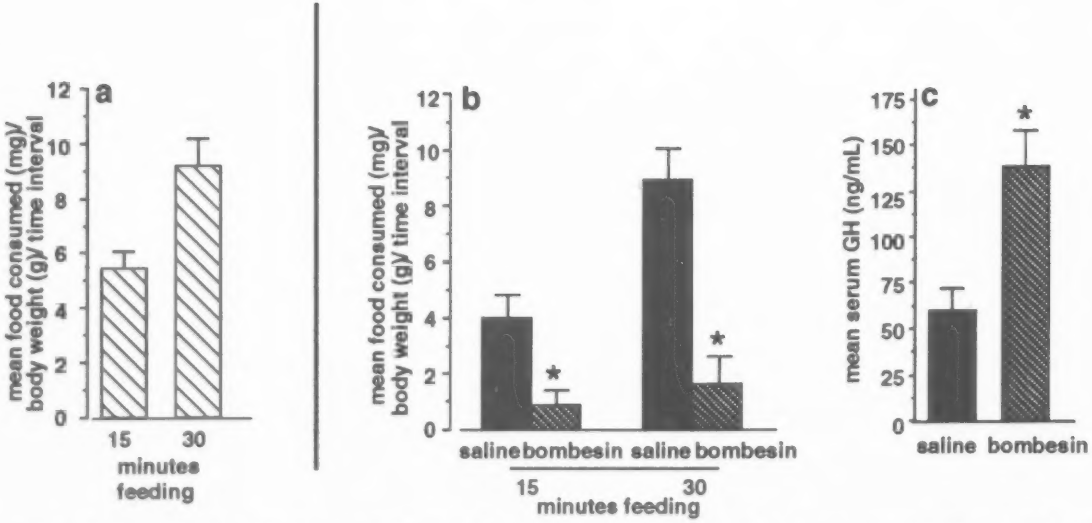

Fig. 3-5. Effects of intraventricular brain injection of bombesin on cumulative food intake in goldfish. Food intake was recorded for 15 and 30 minutes in unhandled fish on day 1 (**a**). On day 2, fish were injected into the third brain ventricle with either bombesin (60 ng/g) (hatched bars) or saline (solid bars) and, at 15 minutes post-injection, cumulative food intake was recorded for 15 and 30 minutes (**b**). Serum growth hormone values (**c**) represent levels at the end of the 30 minute observation period of feeding behavior (n = 9-11 fish/treatment).

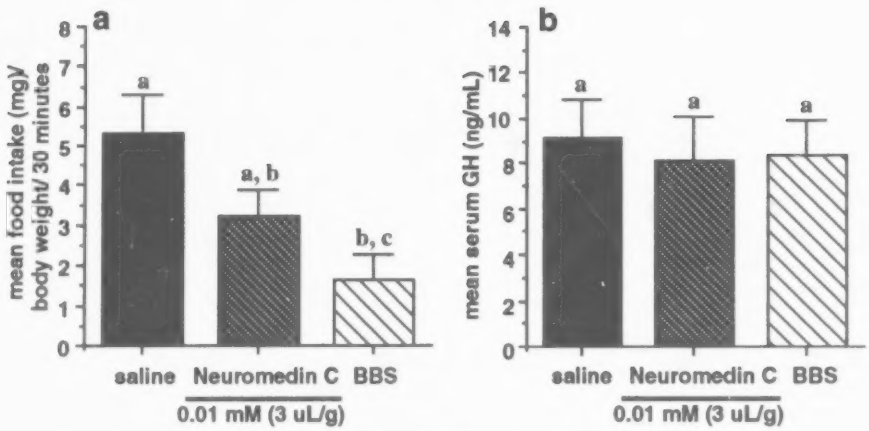

Fig. 3-6. Effects of neuromedin C on feeding behavior and serum growth hormone levels. Groups of fish were injected with saline or an equimolar dose (0.01 mM, 3 ul/g) of either neuromedin C or bombesin. At 15 minutes post-injection cumulative food intake was recorded for 30 minutes (**a**). Serum growth hormone values (**b**) represent levels at the end of 30 minutes of behavioral observation (n = 6- 7 fish/treatment).

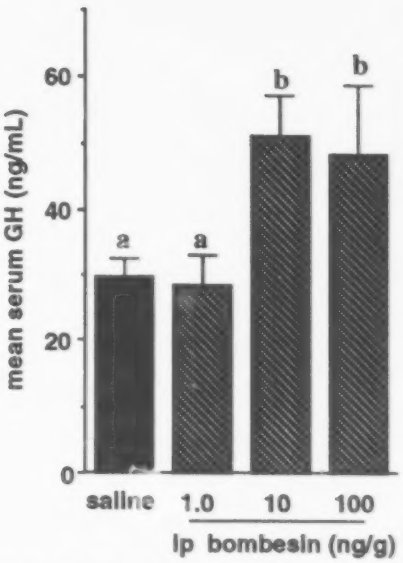

Fig. 3-7. Dose-response of intraperitoneal injection of bombesin on serum growth hormone levels. Groups of fish were injected with either saline (solid bar) or one dose bombesin (1.0, 10, or 100 ng/g) (hatched bars) and sampled at 1.5 hours post-injection (n = 12 fish/treatment).

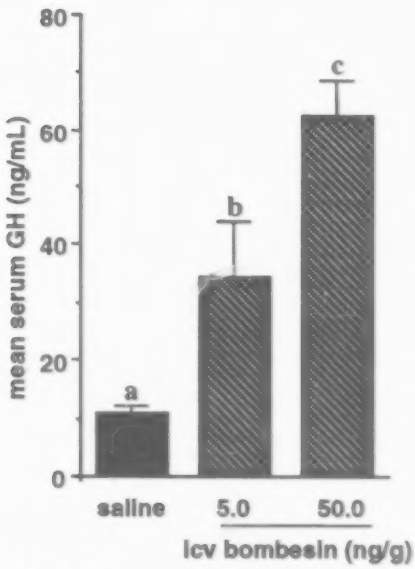

Fig. 3-8. Dose-response of third brain ventricle injection of bombesin on serum growth hormone levels. Groups of fish were injected with either saline (solid bar) or one dose of bombesin (5.0 or 50.0 ng/g) (hatched bars) and sampled at 1.5 hours post-injection (n = 12 fish/treatment).

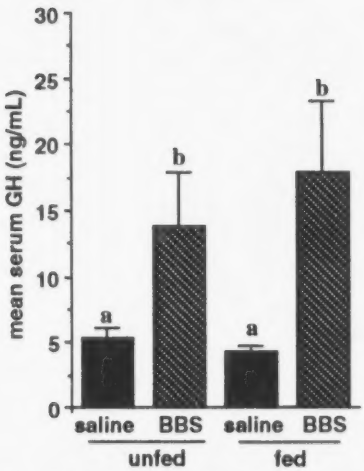

Fig. 3-9. Effects of bombesin on serum growth hormone levels in the presence or absence of food in goldfish. Two groups of fish were injected with 100 ng/g bombesin (BBS; hatched bars), while two groups received saline (solid bars). At 15 minutes post-injection, one group from each treatment received food; at 1.5 hours post-injection all groups of fish were sampled (n = 12 fish/group).

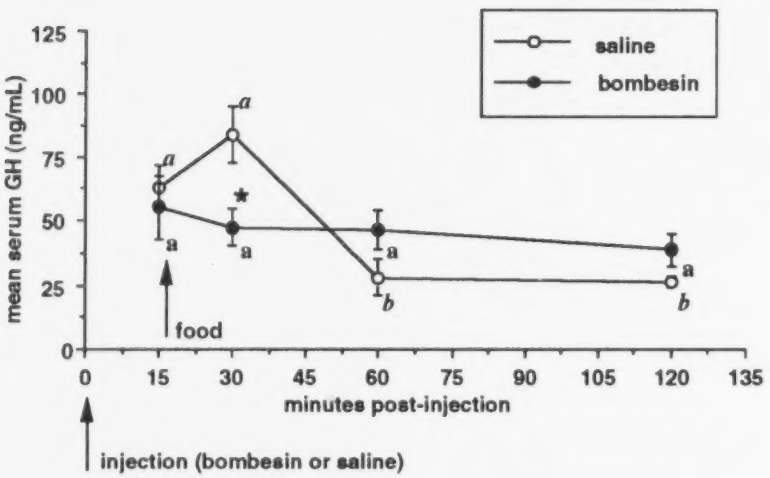

Fig. 3-10. Effects of bombesin on the postprandial circulating serum growth hormone profile in goldfish. Groups of fish were injected at time = 0 with either saline (open circles) or bombesin (50 ng/g) (closed circles). At 15 minutes post-injection one group of saline and one group of bombesin injected fish were sampled. All other groups received food and at 30, 60, and 120 minutes post-injection one group of saline and one group of bombesin-injected fish were sampled (two way anova with interaction, least squared means; lower case = difference between groups of bombesin -injected fish; lower case italics = difference between groups of saline -injected fish; * = difference between groups of bombesin and saline -injected fish; n = 12 fish/group).

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Chapter 4

Bombesin-Like Immunoreactivity in the Goldfish Forebrain and Pituitary: Regulation of Growth Hormone Release by Bombesin in vitro[1]

4.1 Introduction

In mammals, BBS/GRP-like peptides are widely distributed within nerves of the central nervous system and within the gastrointestinal tract (for reviews Panula, 1986, Chapter 1), where they play a fundamental role in the regulation of feeding related activities (for reviews Morley, 1987; McCoy and Avery, 1990). In lower vertebrates such as fish, BBS/GRP-like immunoreactive (IR) material has also been reported in both endocrine cells and nerve fibres of the gastrointestinal tract, including the rainbow trout *Oncorhynchus mykiss* (Holmgren *et al.*, 1982), Atlantic cod *Gadus morhua* (Holmgren and Jonsson, 1988), and members of the cyprinid family (Bjenning and Holmgren, 1988). To our knowledge, however, only one recent study has documented the presence of BBS/GRP-like IR within the central nervous system of fish. In the cartilaginous fish *Scyliorhinus canicula*, BBS-like IR material was reported in ventral forebrain regions of the telencephalon and hypothalamic preoptic area, as well as in the habenular complex (Vallarino *et al.*, 1990). In this fish species, BBS-like IR was also described in a prominent nerve tract within the infundibular floor and in the median eminence, where IR-fibres appeared to form close contact with the vascular system of the pituitary portal system, suggesting a neuroendocrine role for BBS-like peptides (Vallarino *et al.*, 1990).

The role that BBS may play in the regulation of pituitary hormone release in any lower vertebrate has not been investigated, although it is established that BBS/GRP-like peptides directly stimulate the release of growth hormone (GH) and the gonadotropins, luetinizing hormone and follicle-stimulating hormone, in cultured rat pituitary cells at physiological doses (Westendorf and Schonbrunn, 1982; Kentroti *et al*, 1988; Houben

[1]A version of this chapter has been submitted for publication. Himick, BA and Peter, RE. 1994. Neuroendocrinology.

et al., 1990). Such BBS/GRP peptide actions on mammalian pituitary somatotropes are receptor specific, since a highly specific BBS/GRP antagonist is capable of suppressing BBS-induced GH release in a dose-dependent manner (Houben and Denef, 1991b). The presence of [^{125}I] Tyr$_4$-BBS-binding sites on GH cells from the rat anterior pituitary have also been documented (unpublished observations through Houben and Denef, 1991a), further indicating that BBS/GRP peptides have direct actions at the level of the pituitary.

Recently, Himick and Peter (1994) reported that BBS acutely suppressed feeding behavior in the goldfish following either intraperitoneal (ip) or third brain ventricle injection (Chapter 3). Associated with this BBS-induced decrease in food intake were concomitant elevations in circulating serum GH levels. Although the mechanism whereby serum GH levels were increased was unknown, it was suggested that BBS may have acted at the pituitary to modify GH release. Additionally, despite the reported central actions of BBS on feeding behavior following brain ventricle injection (Himick and Peter, 1994; Chapter 3), the presence and localization of BBS/GRP-like peptides within the central nervous system of the goldfish, or any teleost fish for that manner, were not known.

To address such unanswered questions, the focus of this chapter was based on two objectives. To examine if BBS/GRP-like peptides are present in the teleost central nervous system, the first objective investigated the distribution of BBS/GRP-like IR within the goldfish forebrain and pituitary. The second objective was to determine whether BBS is capable of acting directly at the level of the goldfish pituitary to alter GH secretion. Therefore the second part of this chapter focused on the *in vitro* effects of BBS-like peptides on GH release from goldfish pituitary fragments following perifusion with BBS-like peptides. Findings presented in this chapter are the first to provide evidence that BBS/GRP-like peptides exist within the teleost central nervous system and that they may play a role in the regulation of anterior pituitary hormone release in goldfish.

4.2 Materials and Methods

Experimental Animals

Male and female goldfish (25-45 g) were purchased from Ozark Fisheries (Stoutland,

MO) at several times of the year and used in experiments when fish were in a gonadal regressed phase or in a gonadal maturing phase. Fish were maintained under simulated Edmonton natural photoperiod in 1.8 kL tanks which received a constant flow of aerated and dechlorinated city water at 17°C. Fish were fed *ad libitum* twice daily with commercially prepared fish pellets until use for immunohistochemical studies or *in vitro* perifusion experiments.

Immunohistochemistry of BBS/GRP-like Peptides in Goldfish

Tissue Preparation

Brain and pituitary tissues of anesthetized goldfish (0.05% tricaine methanesulfonate; Syndel Laboratories, Vancouver, B.C) were perfused *in situ* via the bulbous arteriosus with ice-cold 0.1 M phospate buffered saline (PBS; pH 7.4), followed by 4% paraformaldehyde containing 0.2% picric acid in PBS, pH 7.4. Both tissues were then removed, post-fixed for 2 h, rinsed in PBS, and cryoprotected by immersion overnight in PBS containing 20% sucrose at 4°C. Brain and pituitary tissues were then frozen in Tissue-Tek (Miles Inc, Elkhart, IN) on dry ice, and placed in a cryostat for temperature equilibration at -17°C. Sections (12-14 μm) were collected on chrome-alum gelatine coated slides. Sections were allowed to dry for a minimum of 2 h and were then rehydrated in PBS containing 0.5 mL/L polyoxyethelene sorbitan monolaurate (Tween 20) (Sigma, St. Louis, MO). To block non-specific binding of the L90 BBS antisera, pituitary sections were incubated with 30% normal goat serum (Calbiochem, La Jolla, CA) prior to administration of primary antisera.

Indirect Immunofluorescence

Following application of normal goat serum, sections were incubated for 18 to 20 h at 4°C in the presence of L90 BBS antisera (1:300). Sections were then rinsed with PBS and exposed in the dark for 60 minutes to goat anti-rabbit Immunoglobulin G which was conjugated to tetramethylrhodamine isothiocyanate (TRITC; 1:100, Sigma) for 60 minutes in the dark. Sections were rinsed with PBS and mounted using carbonate-buffered glycerol/p-phenylenediamine (pH 8.5). Immunoreactive staining was photographed using a Zeiss fluorescence microscope.

Avidin-Biotin Complex Coupled with Peroxidase

Prior to application of normal goat serum, endogenous peroxidase activity was inactivated by incubation of sections in methanol containing 0.3% hydrogen peroxide for 30 minutes. Sections were rinsed in PBS, incubated in normal goat serum, and exposed to L90 BBS antisera (1:300) for 18 to 20 h at 4°C. Sections were then rinsed with PBS and BBS-IR was observed using the avidin-biotin immunoperoxidase method (ABC VectaStain Kit, Vector Labs, Burlingame, CA). Immunoreactivity was visualized as a brown reaction product following incubation with the chromagen diaminobenzidine (Sigma) in 0.05 M TRIS buffer (pH 7.6) containing 0.01% hydrogen peroxide. Immunoreactive staining was photographed using a Ziess light microscope.

Antisera and Specificity Controls

The specificity of rabbit anti-BBS (L90), raised against synthetic BBS-14, has been documented by radioimmunoassay (RIA) previously (Campbell *et al.*, 1989). In addition, routine tests for the specificity of the immunoreactive staining were performed. Staining by L90 was quenched when preabsorbed for 24 h with synthetic BBS-14 (10 nmol/mL diluted antiserum) and gastrin-releasing peptide (GRP) 1-27, but not when preabsorbed with sulfated CCK8 (CCK8-s; 100 nmol/mL) or Substance P (100 nmol/mL). Staining was abolished following the omission of primary antisera and replacement with normal rabbit serum, or following omission of secondary antisera. To identify individual brain nuclei, serial sections were stained with cresyl violet.

Perifusion of Goldfish Pituitary Fragments with BBS

Pituitary Perifusion System

The effects of BBS/GRP-like peptides on the secretion of GH, and another pituitary hormone GtH-II, from goldfish pituitary fragments were examined using a perifusion system described previously (Marchant *et al.*, 1987). Briefly, the fish were anesthetized in 0.05% TMS and killed by spinal section. The pituitaries were quickly removed and cut into fragments (< 0.04 mm^3) using a McIlwain tissue chopper. Each perifusion column contained fragments equivalent to three pituitaries. Fragments were perifused overnight with medium 199 containing Hank's Balanced Salts (Gibco, Grand Island, NY), followed by a 2 h pre-perifusion of Hank's Balanced Salt Solution (HHBSA,

supplemented with 25 mM HEPES and 0.1% BSA). In all experiments, the perifusion flow rate was 15 mL/h, and 5 minute (1.25 mL) fractions were collected using an automatic fraction collector. The samples were stored at -25^{o}C until hormone analyses. The concentration of GtH-II and GH levels in perifusion samples was determined by use of two specific RIAs developed for common carp GtH-II (Peter *et al.*, 1984; Van Der Kraak *et al.*, 1992) and common carp GH (Marchant *et al.*, 1989), as outlined in Chapter 2.

Data Analyses and Statistics

For *in vitro* perifusion experiments, the GH and GtH-II release responses to each pulse of BBS were determined by quantifying the net response above average basal levels. Basal level was defined as the average hormone content in the three fractions collected immediately preceding each pulse (prepulse). Net responses were expressed as a percentage of the prepulse value. A release response was considered to be terminated when hormone content was within one standard error (SEM) of the mean prepulse value. The transformation to percentage of prepulse hormone levels allowed results from several columns to be combined for statistical analysis. Differences in the hormone responses to pulses of BBS were compared by one-way analysis of variance (ANOVA) followed by Student-Newmann Keuls multiple comparison test. For all experiments, significance was at $p \leq 0.05$, and error bars are representative of standard error of measurement (SEM).

Peptides Administered

BBS-14, GRP 1-27, GRP 14-27, neuromedin C, and neuromedin B were purchased from Bachem Bioscience Inc. (Philadelphia, PA), and CCK8-s was purchased from Richelieu Biotechnologies (Saint-Hyacinthe, QC). Substance P was purchased from Sigma. Synthetic sGnRH was purchased from Pennisula Laboratories (Belmont, CA) and was diluted with HHBSA from stock solution (10 µg/20 µL in 0.1 N acetic acid) immediately prior to use in *in vitro* perifusion experiments.

4.3 Results

4.3.1 BBS/GRP-like IR in the Goldfish Pituitary and Brain

4.3.1.1 Pituitary

Of all goldfish nervous tissue regions examined for BBS/GRP-like IR material, the neurointermediate lobe (NIL) consistently contained the highest density of BBS/GRP-like IR material. Nerve fibres containing BBS/GRP-like IR branched throughout the NIL (Fig. 4-1 a and b); these IR fibres extended to the boundary between the NIL and the pars distalis (PD) of the anterior pituitary. Few BBS/GRP-like IR fibres were also detected in the hypophysial stalk region entering the posterior pituitary, indicating that BBS/GRP-like peptides within the NIL have an extrinsic origin. On occasion, a few BBS/GRP-like IR fibres and IR perikarya were detected in the pars distalis (Fig. 4-1 a). Staining for BBS/GRP-like IR material was completely quenched in the NIL and anterior pituitary following preabsorption with either BBS-14 or GRP 1-27 (Fig. 4-1 c and d), but not with Substance P or CCK8-s.

4.3.1.2 Forebrain

Telencephalon

Few, fine-beaded BBS/GRP-like IR fibres were detected throughout ventral regions of the telencephalon, including the area ventralis telencephali pars ventralis (Vv), area ventralis telencephali pars lateralis (Vl), and area ventralis telencephali pars dorsalis (Vd) (Fig. 4-2 A). On occasion, thin-beaded BBS/GRP-like IR fibres were observed in the dorsal telencephalon.

Diencephalon

BBS/GRP-like IR fibres were detected in ventral hypothalamic preoptic regions, including the nucleus entopeduncularis (NE), nucleus preopticus periventricularis (NPP), and nucleus preopticus (NPO). In some instances, BBS/GRP-like IR perikarya were also localized in the NPO and NPP (Fig. 4-2 B and C). BBS/GRP-like IR fibres appeared to increase in density in more ventro-posterior regions of the hypothalamus and in the hypothalamic inferior lobes, including the nucleus lateral tuberis pars anterioris (NLTa) and nucleus lateral tuberis pars posterioris (NLTp), nucleus anterior tuberis (NAT), nucleus recessus lateralis (NRL) and nucleus recessus posterioris (NRP), and the

nucleus diffusus lobi inferioris (NDLI) (Fig. 4-2 E, F; Fig. 4-3 d). In addition, the nucleus glomerulosus (NG) and the nucleus diffusus tori lateralis (NDTL) contained few fine-beaded BBS/GRP-like IR fibres (Fig. 4-2 E, F). Periventricular regions of the NLT, NAT, NRL, and NRP occasionally contained IR cell bodies (Fig. 4-2 E, F; Fig. 4-3 c).

BBS/GRP-like-IR fibres and IR perikarya were localized within dorso-posterior regions of the diencephalon, including the nucleus habenularis (NH) and the nucleus rotundus (NR) (Fig. 4-2 E; Fig. 4-3 b and c). Finally, fine-beaded BBS/GRP-like IR fibres and IR perikarya were detected within several thalamic nuclei and the optic tectum (OTec) (Fig. 4-2 D and E). BBS/GRP-like IR material was also observed in bilateral patches within the inner dorsal lining of the ventricle at the level of the dorso-anterior thalamus (Fig. 4-3 e).

BBS/GRP-like IR fibres were also detected in areas more posterior to the goldfish forebrain, such as the the nucleus interpeduncularis (NIp) of the midbrain (Fig. 4-3 f) and in the facial and vagal lobes of the hindbrain (Fig. 4-2 H).

4.3.2 Effects of BBS/GRP-like peptides on goldfish pituitary GH and GtH-II release in vitro

4.3.2.1 BBS repeated pulses (0.1 nM or 1000 nM) Fig. 4-4 represents the mean GH (a, b) and GtH-II (d, e) release responses from pituitary fragments of gonadal maturing goldfish which were exposed to four repeated pulses of either 0.1 or 1000 nM BBS. The effects of repeated BBS challenges on GH and GtH-II secretion when quantified as the mean total hormone release per pulse (expressed as a percent of basal prepulse levels) are shown in (c) and (f), respectively. Initial challenge of 0.1 nM BBS (pulse 1) resulted in a net average GH release response of 91% over basal, while the net GtH-II release response was increased 68% above basal. Initial challenge of 1000 nM BBS resulted in a net GH release response of 176% over basal, while the response of GtH-II secretion to 1000 nM BBS was increased to 159% over basal. The second pulse of 0.1 nM BBS stimulated a greater GH release response (197% over basal) compared to initial challenge, while the GH release-reponses in the third and fourth pulses were of similar magnitude to the first pulse (63% and 103%, respectively). Likewise, the second pulse of 0.1 nM

BBS resulted in a greater GtH-II release response (142%) greater than the initial challenge; the GtH-II release responses to the third and fourth pulses were significantly lower than to the first and second pulses (12% and 11%, respectively). Repeated pulses of 1000 nM BBS produced a significant decrease in the GH release response. While the GH release response to the second pulse of 1000 nM BBS was similar to the response obtained in the intial challenge, the GH release response to the third and fourth pulses were significantly smaller compared to the responses to the first and second pulses (pulse 3 , 60%; pulse 4, 94%). The GtH-II release responses to the second, third and fourth pulses of 1000 nM BBS were significantly smaller than to the first pulse. In all columns, a five minute control pulse of sGnRH at the end of the experiment stimulated both GtH-II and GH release.

4.3.2.2 BBS repeated pulses (10 nM or 100 nM) Fig. 4-5 represents the mean GH (a, b) and GtH-II (d, e) release responses of pituitary fragments from gonadal regressing goldfish exposed to three repeated pulses of either 10 or 100 nM BBS. The net hormone release responses per pulse (expressed as a percent of basal prepulse levels) are also shown (Fig. 4-5 c and f). Three successive challenges of 10 nM BBS did not result in significant differences between net responses during each pulse of either GH or GtH-II. Similarly, repeated challenge of 100 nM BBS resulted in no differences in the net GH or GtH-II release responses between pulses. In all columns, a five minute control pulse of sGnRH at the end of the experiment stimulated both GtH-II and GH release.

4.3.2.3 BBS repeated pulses (0.1 nM) Fig. 4-6 represents the GH (a) and GtH-II (b) release responses from pituitary fragments of gonadal regressed goldfish exposed to five repeated pulses of 0.1 nM BBS. Fig. 4-6 (c and d) represent the net hormone release responses per pulse for GH and GtH-II, respectively. Initial challenge of 0.1 nM BBS resulted in a GH release response of 9.0% above basal. The release responses were elevated with subsequent pulses of BBS and at the fifth peptide challenge, the net GH release response was significantly increased to 82% above basal relative to the first or second pulse. Repeated challenge of 0.1 nM BBS induced GtH-II release responses of similar magnitude. In all columns, a five minute control pulse of sGnRH at the end of the experiment stimulated both GtH-II and GH release.

4.3.2.4 BBS repeated pulses and BBS-like peptides (100 nM) Fig. 4-7 represents the GH release responses from pituitary fragments of gonadal regressed goldfish when exposed to five repeated pulses of either 100 nM BBS (a) or 100 nM of the BBS-like peptides, neuromedin B (NMB), GRP 1-27, GRP 14-27, neuromedin C (NMC), or BBS (b). Fig. 4-8 (a and b) represents the GtH-II release responses from fragments following perifusion of BBS and BBS-like peptides. Fig. 4-7 (c and d) and Fig. 4-8 (c and d) represent the net hormone release responses per pulse for GH and GtH-II, respectively. Initial challenge of 100 nM BBS resulted in a GH release response of 97% above basal; the release responses in subsequent pulses were significantly decreased. Initial challenge of 100 nM BBS induced a GtH-II release response of 126% above basal; the release response was also significantly decreased following perifusion of pulse 3 (22% above basal). However, an increased GtH-II release responsiveness was evident during pulse 5 (114% above basal).

Perifusion of the BBS-like peptides resulted in GH and GtH-II release responses which were similar in magnitude between peptide challenge. In all columns, a five minute control pulse of sGnRH at the end of the experiment stimulated both GtH-II and GH release.

4.3.2.5 BBS-like peptides (100 nM) Fig. 4-9 represents the GH (a) and GtH-II (b) release responses from pituitary fragments of gonadal late recrudescent goldfish when exposed to four repeated pulses of either 100 nM NMC, GRP 14-27, GRP 1-27, or BBS. Fig. 4-9 (c and d) represents the net hormone release responses of GH and GtH-II following peptide challenge, respectively. Perifusion of BBS resulted in a significant elevation in both GH and GtH-II release when compared to responses obtained during pulses of NMC or GRP 14-27, respectively. In all columns, a five minute control pulse of sGnRH at the end of the experiment stimulated both GtH-II and GH release.

4.4 Discussion

Data in this chapter demonstrate that BBS/GRP-like IR material exists in the goldfish forebrain and pituitary. These studies are the first to document the distribution of

BBS/GRP-like IR material in the central nervous system of a teleost. Fine-beaded, sparsely distributed BBS/GRP-like IR fibres and, in some instances IR perikarya, were localized predominantly to ventral nuclei of the goldfish telencephalon and hypothalamus. In particular, BBS/GRP-like IR was detected in regions associated with the preoptic hypothalamus (NE, NPP, and NPO) and the ventro-posterior hypothalamus and hypothalamic inferior lobes (NRL, NRP, NAT, NLT). BBS/GRP-like IR fibres and few IR cell bodies were also detected in the NR, NH, and OTec of the dorsal posterior diencephalon. This pattern of staining within the goldfish forebrain resembles that of the distribution for BBS-like IR reported in the brain of the cartilaginous fish, *S. canicula* (Vallarino *et al.*, 1991). In *S. canicula*, a prominent BBS-like IR fibre system was detected in the ventral telencephalon and diencephalon. In addition, fibres immunoreactive for BBS were localized amongst structures of the dogfish hypothalamo-hypophysial neurosecretory system, including the magnocellular cells of the NPO, hypothalamic fibres, and terminals entering the median eminence. Based on the anatomical locations of BBS-like IR material in *S. canicula*, it was suggested that BBS-like peptides may play a role in the regulation of pituitary hormone secretion. In the present study, BBS/GRP-like IR fibres and IR perikarya were also detected in the NPO and NPP, two nuclei which project peptidergic fibres directly into the goldfish anterior pituitary and NIL (Peter and Fryer, 1983). Like *S. canicula*, the presence of BBS/GRP-like IR within the NPO and NPP, NLT, in the hypophysial stalk, and subsequently in neurons throughout the NIL and occasionally in the anterior pituitary, strongly indicates that BBS peptides play some role in the regulation of pituitary hormone secretion in the goldfish. While the effects of BBS on hormones from the goldfish NIL are unknown, peripheral or third brain injection of BBS into goldfish alters circulating serum GH levels (Chapter 3; Himick and Peter, 1994), and perifusion of goldfish pituitary fragments with BBS (present chapter) results in the release of both GH and GtH-II.

In addition to the presence of BBS/GRP-like IR material in the hypothalamic preoptic regions and pituitary of the goldfish, BBS/GRP-like IR was detected in several ventro-posterior regions of the hypothalamus and in the hypothalamic inferior lobes. It has been established that both these areas regulate feeding behavior in fish, based primarily on earlier research involving electrical stimulation of specific posterior hypothalamic nuclei (Demski and Knigge, 1971; Demski, 1973), as well as anatomical and histological

studies in several teleost species which illustrate medial forebrain bundle connections and gustatory input into the hypothalamic inferior lobes (Herrick, 1905; Crosby and Showers, 1969; Finger, 1978; for review Demski, 1981; Demski, 1983). For example, if bilateral lesions are placed in the lateral regions of the hypothalamus or the hypothalamic inferior lobes of goldfish, a reduction in feeding behavior occurs (Roberts and Savage, 1978). Alternatively, if areas in the ventro-posterior hypothalamus, such as the NRL and NPGL, are electrically stimulated an increase in feeding activity results (Savage and Roberts, 1975). Recently, we reported that BBS injection into the third brain ventricle of the goldfish results in an acute suppression in food intake (Chapter 3; Himick and Peter, 1994). However, the sites of action of BBS which mediated this decrease in feeding behavior were not known. Furthermore, at that time the presence of BBS/GRP-like peptides within the goldfish central nervous system was unknown. The present anatomical findings of BBS/GRP-like IR material in the fish brain hypothalamic feeding center, as well as BBS/GRP-like IR localized in periventricular structures within the ventro-posterior hypothalamus (NRL, NRP and NPPv) support a role for BBS peptides in the central regulation of feeding behavior in the goldfish.

It was also reported that concomitant with BBS-induced feeding suppression following either peripheral or third brain ventricle injection in goldfish were elevations in circulating levels of serum GH (Chapter 3; Himick and Peter, 1994). While the mechanism whereby BBS stimulated serum GH levels was unknown, we had speculated that BBS may have acted at the level of the goldfish pituitary to alter hormone secretion. In the present study, we present evidence that BBS is capable of modifying the release of GH, as well as GtH-II, by direct actions at the level of the pituitary.

Data indicate that when pituitary fragments were initially challenged with either 0.1 nM, 10 nM, 100 nM, or 1000 nM BBS, an increase in the release of both GH and GtH-II occurred. However, while successive challenge of a low dose of BBS (0.01 nM or 0.1 nM) resulted in a sensitization in the GH, repeated administration of high doses of 1000 nM BBS (and 100 nM in gonadally regressed fish) produced a reduction in the sensitivity in the GH and GtH-II release responses. It is likely that with repeated challenge of BBS to fragments, changes may occur at the level of the receptor resulting in a desensitized hormone response to repeated pulses of high dosages of BBS, and a sensitized hormone response following BBS challenge at low doses. Furthermore, data

presented here indicate that GH responsivness is more susceptible to alteration following repeated BBS exposure than the GtH-II response when fragments from maturing fish are challenged. Altered sensitivity of both GH and GtH-II release responses from goldfish pituitary fragments has been reported following repeated exposure to other neuropeptides, including neuropeptide Y (Peng *et al.*, 1990) and cholecystokinin (Himick *et al.*, 1993; Chapter 7). While the mechanism whereby altered hormonal sensitivity to BBS at the pituitary cell level is unknown, it is clear that the hormonal desensitization response was not due to depletion of the releasable pools of stored hormone from the somatotropes and gonadotropes of the pituitary fragments, since GH and GtH-II release responses were still obtained following the control pulse of sGnRH at the end of experiments.

The present results of GH and GtH-II release following pituitary fragment challenge with BBS-related peptides are more difficult to interpret. Both desensitization and sensitization GH and GtH-II responses can occur following repeated exposure of fragments to BBS (refer to control 100 nM pulses of BBS conducted in parallel to perifusion of BBS-related peptide challenge in Fig. 4-7). Since BBS/GRP-like peptides and NMB are capable of binding with high affinity to the BBS receptor in the goldfish CNS (refer to Chapter 7), the amount of GH and GtH-II release following peptide perifusion may in fact not be a true representation of the stimulatory capacities of BBS-related peptides on GH and GtH-II release. Nonetheless, data presented here demonstrate that BBS-like peptides are capable of releasing both GH and GtH-II from fragments of gonadal recrudescent and regressed fish.

The present findings that BBS can directly release GH and GtH-II from the pituitary of the goldfish are the first studies to provide evidence that a neuroendocrine function may exist for BBS-like peptides in lower vertebrates. In mammals, however, BBS and GRP stimulate the release of both GH and gonadotropins following incubation with cultured pituitary cells. An acute release of GH has been shown following incubation of rat pituitary cells with physiological doses of BBS (Westendorf and Schonbrunn, 1982), while a dose-dependent stimulation of GH release has been reported after incubation of cultured pituitary cells with concentrations of GRP ranging from 10^{-9} to 10^{-6}M or BBS at doses of 0.1 nM to 10 nM (Kentroti and McCann, 1985).

In the goldfish, BBS/GRP-like IR was consistently localized in nerve fibres within the NIL, the hypophysial stalk, and in NLT regions of the goldfish hypothalamus. Only in rare instances were BBS/GRP-like IR fibres and IR cells detected in the anterior goldfish pituitary. These findings are consistent with the distribution of BBS/GRP-like IR in the pituitaries of several mammalian species including the rat, guinea pig, cat, dog, pig, cow, monkey, and human, where BBS/GRP-like IR material was reported in the intermediate and/or posterior lobes (Houben and Denef, 1991a; Steel *et al.*, 1992). Unlike the goldfish however, BBS/GRP-like IR was also consistently localized in somatotropes of the mammalian anterior pituitary (Steel *et al.*, 1992). Despite the presence of BBS/GRP-like IR primarily in the NIL of the goldfish, BBS is still capable of acutely modifying GH levels when injected peripherally or into the third brain ventricle (Himick and Peter, 1994; Chapter 3), or when perifused over goldfish pituitary fragments *in vitro* (present chapter). It remains possible that BBS/GRP-like peptides of NIL origin may be released into the pituitary blood supply, which may in turn allow the peptide to come in contact with somatotropes of the anterior pituitary. Alternatively, BBS-like peptides may interact with known physiological regulators of GH, such as somatostatin (SRIF) and/or growth-hormone releasing factor (GRF), both of which may still be present and functional in nerve terminals of our pituitary fragment system. In mammals, BBS/GRP-like peptides have been reported to alter pituitary GH secretion through interactions with GRF and SRIF *in vivo* and *in vitro* (Kabayama *et al.*, 1984; Kentroti *et al.*, 1988). More recently, it has been reported that BBS/GRP-like peptides likely regulate the release of LH *in vivo* through actions at a level higher than at the rat pituitary (Pinski *et al.*, 1992). Finally, the lack of consistent BBS/GRP-like IR staining of cells in the goldfish anterior pituitary does not indicate that BBS/GRP-like peptides are not always present in this pituitary region. As has been documented in the fish gastrointestinal tract (Conlon *et al.*, 1987; Bjenning *et al.*, 1990; Jensen and Conlon, 1992), more than one form of BBS/GRP-like peptide may exist in the goldfish pituitary, and as such the specificity of the L90 antisera used may have detected IR material present only in the NIL and within a specific anterior pituitary cell type. In the present study, it is unclear which anterior pituitary cell it is that exhibits BBS/GRP-like IR following use of L90 antisera.

In conclusion, these studies provide initial evidence that BBS/GRP-like IR material

exists within the central nervous system of teleost fish. The anatomical distribution of BBS/GRP-like IR in goldfish hypothalamic nuclei that are a part of the fish brain "feeding center" supports previous findings in Chapter 3 that BBS plays a role in the central regulation of food intake in the goldfish. Additionally, the distribution of BBS/GRP-like IR material in hypothalamic regions which contain neurons known to influence anterior pituitary hormone secretion, as well as the presence of BBS/GRP-like IR in the NLT, hypophyseal stalk and pituitary, indicates that BBS/GRP-like peptides are likely involved in the regulation of pituitary hormone release in the goldfish. The direct stimulation of GH and GtH-II secretion following perifusion of goldfish pituitary fragments with BBS further suggests that BBS is capable of functioning as a neuroendocrine modulator in fish. Direct actions of BBS at the level of the goldfish pituitary may be one mechanism whereby BBS-induced feeding suppression in the goldfish mediates concomitant alterations in circulating serum GH levels (Himick and Peter, 1994; Chapter 3).

Fig. 4-1. Distribution of BBS/GRP-like IR in the goldfish pituitary.

(a) Rhodamine-labeled BBS/GRP-like IR fibres within the neurointermediate lobe (NIL) of the goldfish pituitary. BBS/GRP-like IR was also detected in the hypophyseal stalk entering the pituitary gland (small arrow), 40X; scale bar = 280 μm, PD = pars distalis, **(b)** BBS/GRP-like IR neurons forming net-like connections within the NIL, (rhodamine-labeled); 400X, scale bar = 30 μm, **(c)** BBS/GRP-like IR fibres in the NIL (ABC); 400X, scale bar = 30 μm, **(d)** Adjacent section to (c) showing abolished BBS/GRP-like IR staining in NIL following preabsorption with GRP 1-27, (ABC); 400X, scale bar = 30 μm.

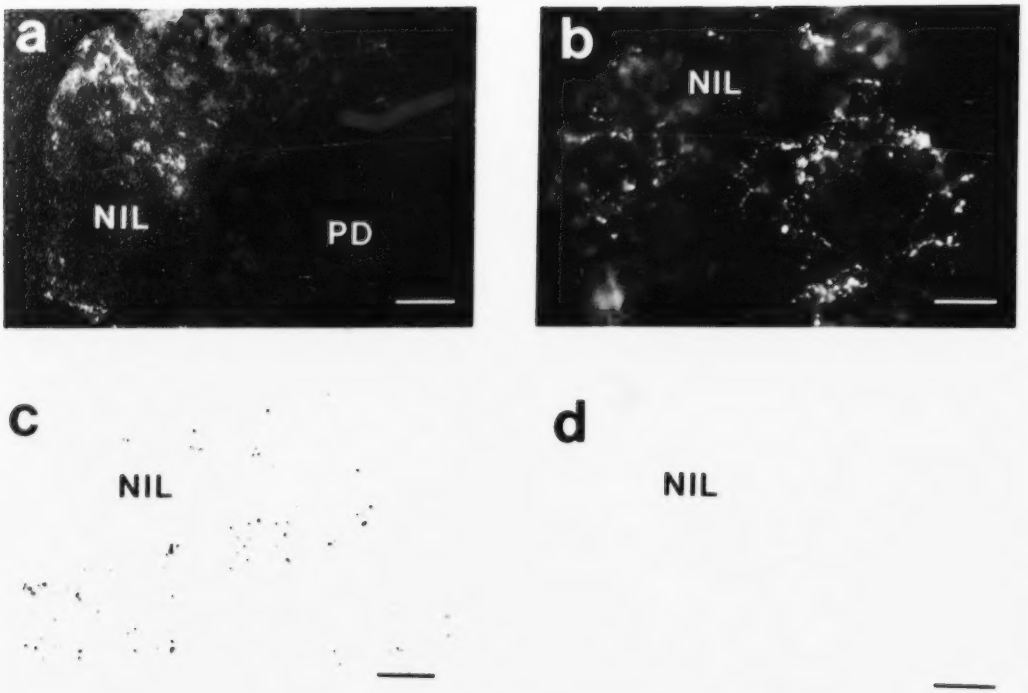
a
NIL
PD
b
NIL
c
NIL
d
NIL

Fig. 4-2 (A-E). Distribution of BBS/GRP-like IR in the goldfish forebrain. Distribution of BBS/GRP-like IR in the goldfish forebrain. Fine dots represent BBS/GRP-like IR nerve fibre terminals; larger dark circles represent BBS/GRP-like IR perikarya. Density of fine dots and dark circles indicate the abundance of BBS/GRP-like IR material within specific brain regions. Intervals between successive sections are A-B: 800 μm; B-C: 800 μm; C-D: 800 μm: D-E: 3000 μm.

List of abbreviations are as follows: AP (area pretectalis); C (cerebellum); CM (Corpus mamillare); Dc (area dorsalis telencephali pars centralis); Dd (area dorsalis telencephali pars dorsalis); Dl (area dorsalis telencephali pars lateralis); Dl_v (area dorsalis telencephali pars lateralis ventralis); Dm (area dorsalis telencephali pars medialis); FL (facial lobe); HOC (horizontal commissure); HAP_v (nucleus anterioris periventricularis); MT (midbrain tegmentum); NAH (nucleus anterioris hypothalami); NAT (nucleus anterior tuberis); NCH (nucleus cerebellosus hypothalami); NDL (nucleus dorsolateralis thalami); NDLI (nucleus diffusus lobi inferioris); NDM (nucleus dorsomedialis thalami); NDTL (nucleus diffusus tori lateralis); NE (nucleus entopeduncularis); NG (nucleus glomerulosus); NH (nucleus habenularis); NLT (nucleus lateral tuberis); NLT_l (nucleus lateral tuberis pars lateralis); NLT_p (nucleus lateral tuberis pars posterioris); NP (nucleus pretectalis); NPG_l (nucleus preglomerulosus pars lateralis); NPG_m (nucleus preglomerulosus pars medialis); NPO (nucleus preopticus); NPP (nucleus preopticus periventricularis); NPP_v (nucleus posterioris periventricularis); NPT (nucleus posterioris tuberis); NRL(nucleus recessus lateralis); NRP (nucleus recessus posterioris); NT (nucleus tenia); NTP (nucleus posterioris thalami); NVM (nucleus ventromedialis thalami); OC (optic chiasma); OT (optic tract); OTec (optic tectum); PC (posterior commissure); PIT (pituitary); SD (saccus dorsalis); Vd (area ventralis telencephali pars dorsalis); Vl (area ventralis telencephali pars lateralis); Vv (area ventralis telencephali pars ventralis); Vs (area ventralis telencephali pars supracommissuralis) (nomenclature after Peter and Gill, 1975).

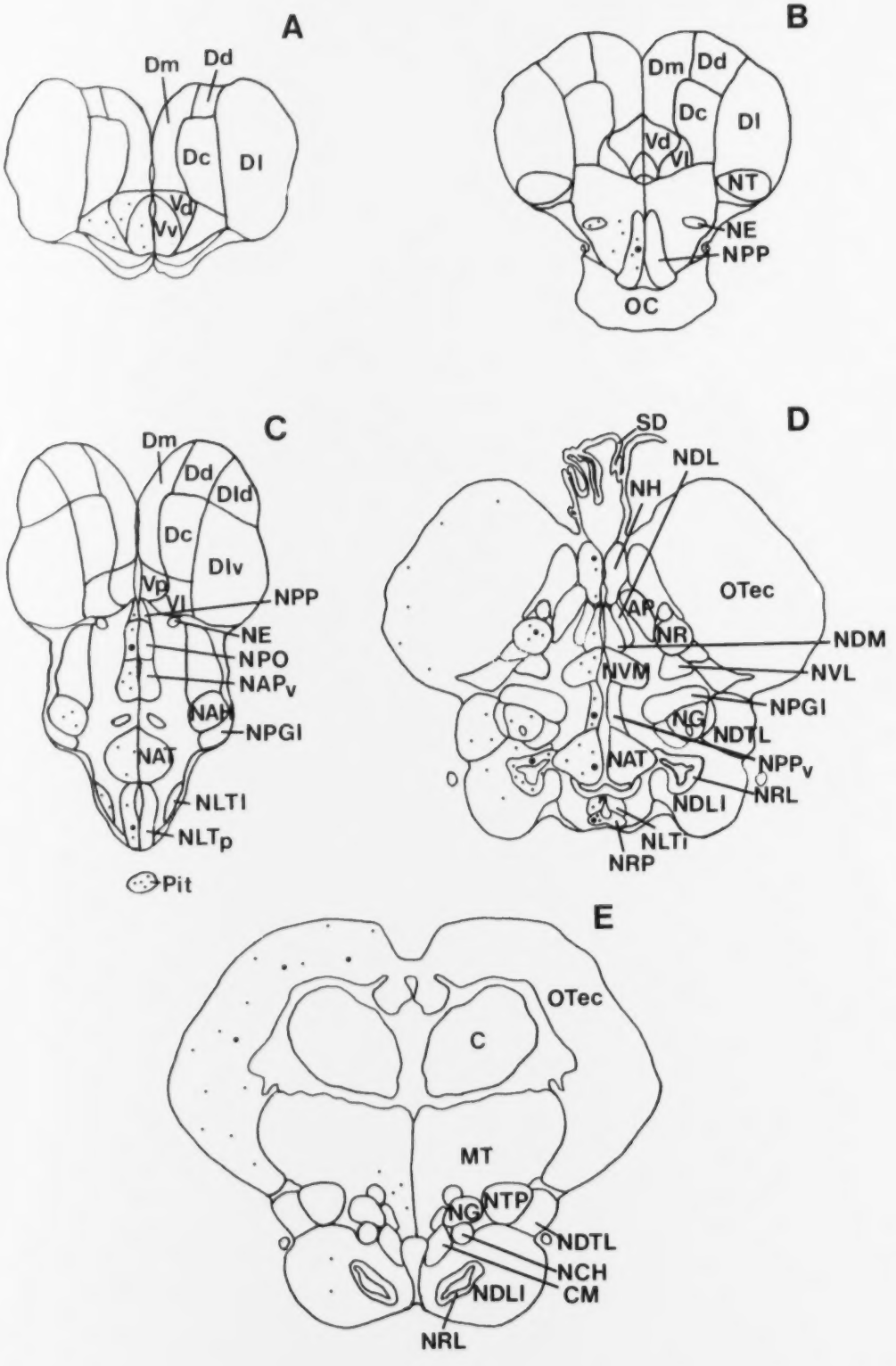
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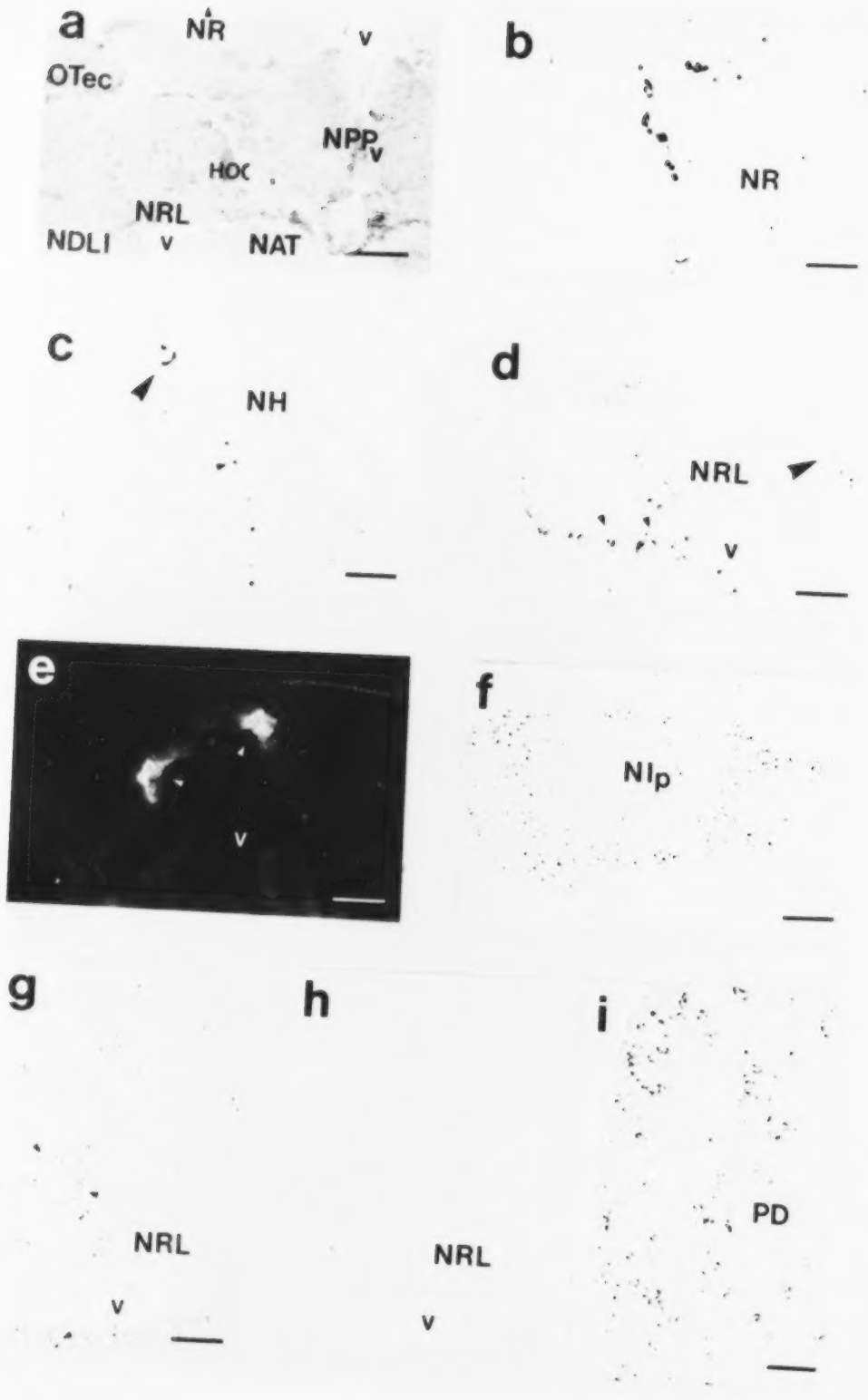
Fig. 4-3. BBS/GRP-like IR in the hypothalamic feeding area of the goldfish. **(a)** Ventro- posterior inferior lobe of goldfish hypothalamus stained with cresyl violet to indicate nuclear areas of NRL, NPPv, NDLI, NR, and OTec where BBS/GRP-like IR material was localized; refer to Fig. 2 for list of abbreviations, v = ventricle; 40X; scale bar = 275 μm, **(b)** BBS/GRP-like IR fibre within the NR of the caudal hypothalamus (ABC), 400X, scale bar = 30 μm, **(c)** BBS/GRP-like IR fibre (small arrow) and IR cell body (arrow) in the NH (ABC), 400X, scale bar = 30 μm, **(d)** BBS/GRP-like IR fibres in the dorsal (arrow) and periventricular regions (small arrows) of the NRL, (ABC), 100X, scale bar = 80 μm, **(e)** Rhodamine-labeled BBS/GRP-like material within the inner dorsal ventricular lining (arrows), 100X, scale bar = 80 μm, **(f)** Dense BBS/GRP-like IR fibres in the midbrain NIp (ABC), 400X, scale bar = 30 μm, **(g)** BBS/GRP-like IR fibres in the posterior NRL (small arrows) (ABC), 250X, scale bar = 55 μm, **(h)** Adjacent section to (g) showing abolished BBS/GRP-like IR staining in the NRL following preabsorption with BBS 14 (ABC), 250X; **(i)** Cells within the pars distalis containing BBS/GRP-like material (ABC), 250X, scale bar = 55 μm.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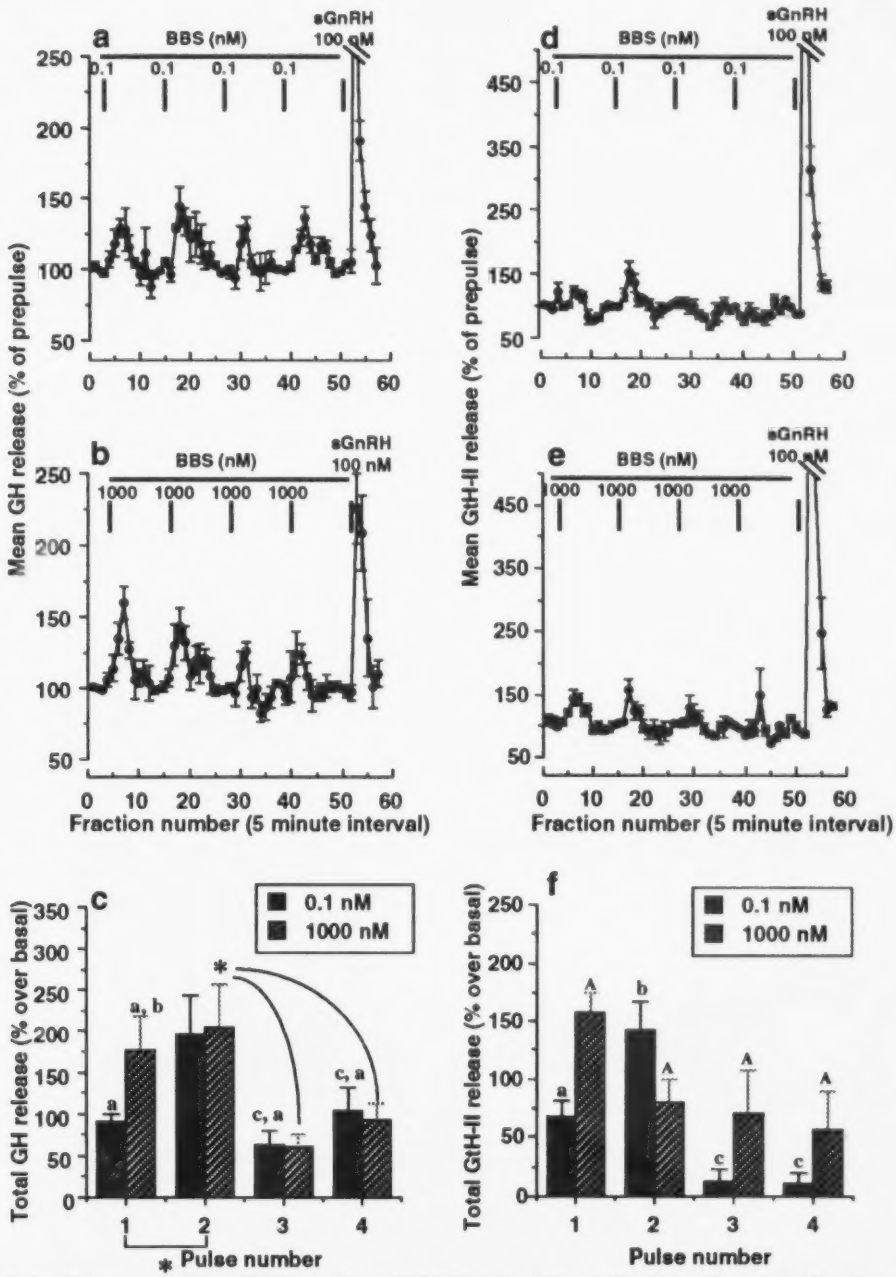

Fig. 4-4. Release profiles of GH (a, b) and GtH-II (d, e) in perifusion columns following repeated challenge with 0.1 nM or 1000 nM BBS. Pituitary fragments from sexually gonadal mature goldfish were exposed to four 5 minute pulses of either 0.1 nM or 1000 nM BBS given at 55-minute intervals. At the end of the experiment, a 5 minute pulse of 100 nM sGnRH was administered as a control. Black bars represent application of peptide. The effects of BBS on total GH and GtH-II release when expressed as the mean total hormone released per pulse are summarized in (c) and (f), respectively (n = 4 columns/dose; mean hormone basal levels prior to peptide challenge, GH 3.74 ng/mL; GtH-II 1.63 ng/mL) Asterisk represents significance between groups with Student's t-test, $p < 0.05$.

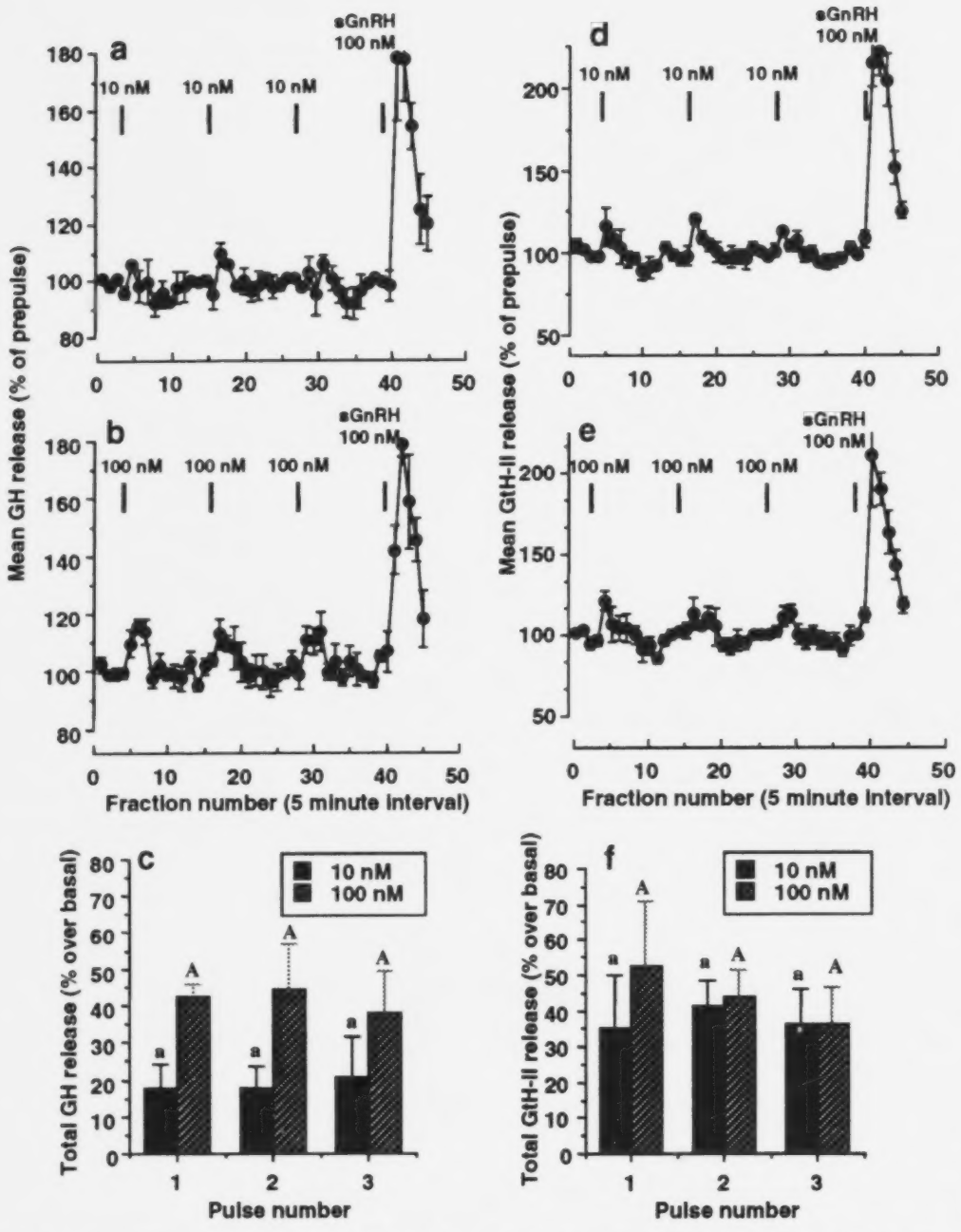

Fig. 4-5 Release profiles of GH (a, b) and GtH-II (d, e) in perifusion columns following repeated challenge with 10 nM or 100 nM BBS. Pituitary fragments from gonadal regressing goldfish were exposed to three 5 minute pulses of either 10 nM or 100 nM BBS given at 55-minute intervals. At the end of the experiment, a 5 minute pulse of 100 nM sGnRH was administered as a control. Black bars represent application of peptide. The effects of BBS on total GH and GtH-II release when expressed as the mean total hormone released per pulse are summarized in (c) and (f), respectively (n = 4 columns/dose; mean hormone basal levels prior to peptide challenge, GH 8.91 ng/mL; GtH-II 2.01 ng/mL).

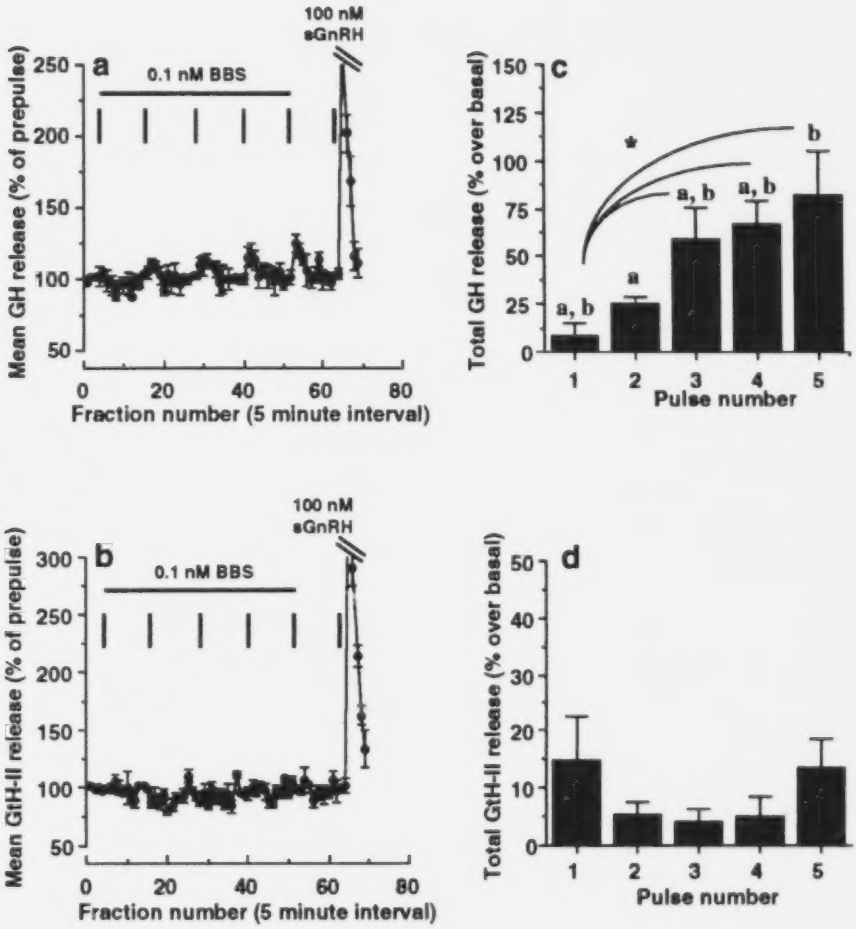

Fig. 4-6. Release profiles of GH (a) and GtH-II (b) in perifusion columns following challenge with repeated pulses of 0.1 nM BBS. Pituitary fragments from gonadal regressed goldfish were exposed to five 5 minute pulses of repeated pulses of 0.1 nM BBS given at 55-minute intervals. At the end of the experiment, a 5 minute pulse of 100 nM sGnRH was administered as a control. Black bars represent application of peptide. The total amount of GH and GtH-II released during each pulse is represented in **(c)** and **(d)**, respectively (n = 4 columns/dose; mean hormone basal levels prior to peptide challenge, GH 8.8 ng/mL). Asterisk represents significance between groups with Student's t-test, $p < 0.05$.

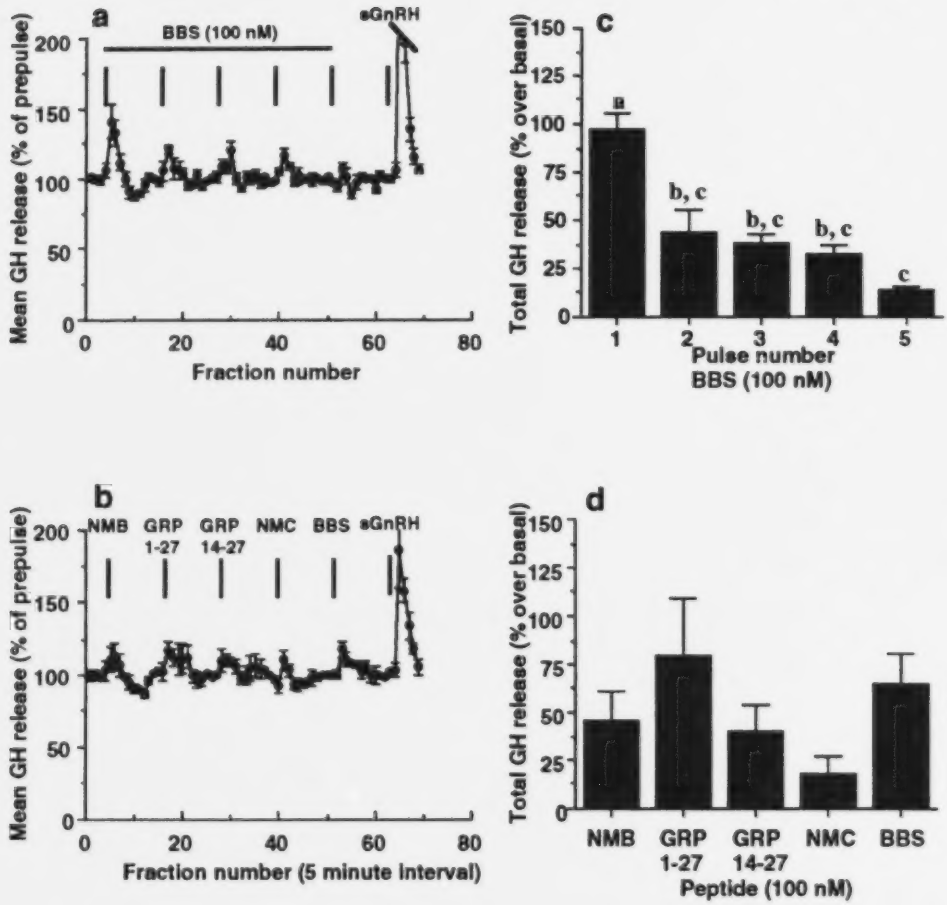

Fig. 4-7. Release profiles of GH in perifusion columns following challenge with BBS and BBS-like peptides. Pituitary fragments from gonadal regressed goldfish were exposed to five 5 minute pulses of 100 nM BBS (**a**) or 100 nM of one of five BBS-like peptides (**b**) given at 55-minute intervals. At the end of the experiment, a 5 minute pulse of 100 nM sGnRH was administered as a control. Black bars represent application of peptide. The effects of BBS and BBS-like peptides on total GH release when expressed as the mean total hormone released per pulse are summarized in (**c**) and (**d**), respectively (n = 4 columns/dose; mean hormone basal levels prior to peptide challenge, GH 12.0 ng/mL).

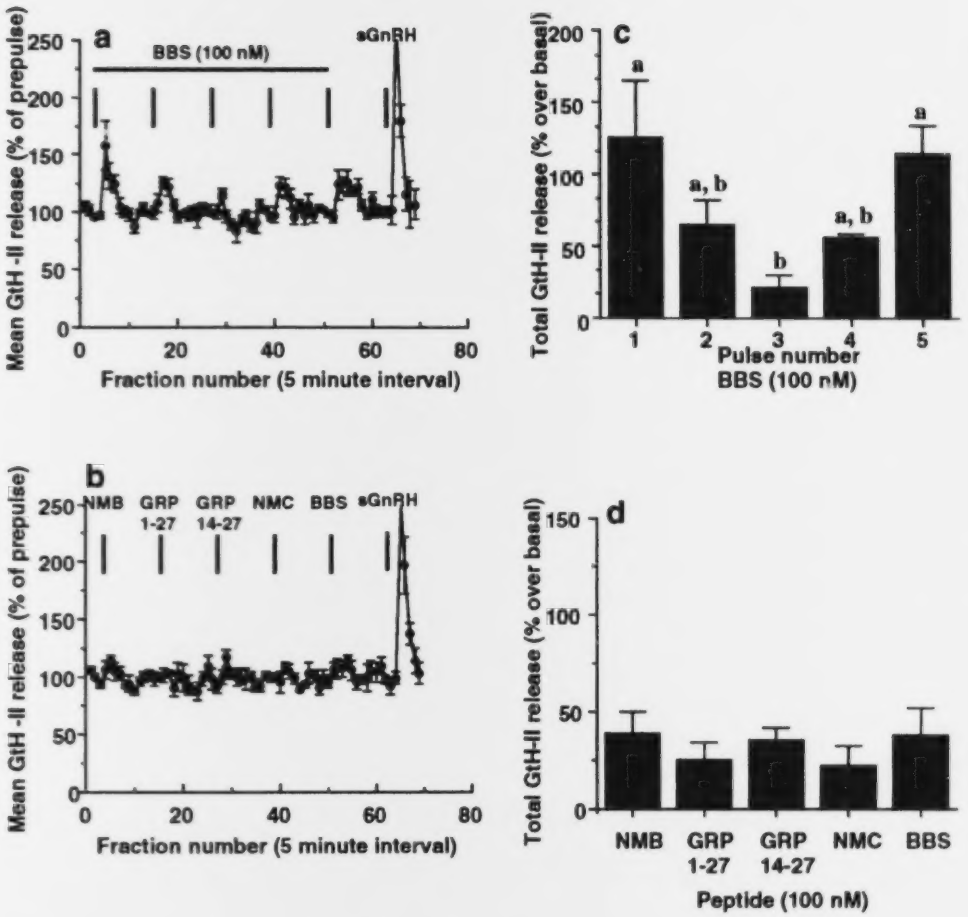

Fig. 4-8. Release profiles of GtH-II in perifusion columns following challenge with BBS and BBS-like peptides. Pituitary fragments from gonadal regressed goldfish were exposed to five 5 minute pulses of 100 nM BBS **(a)** or 100 nM of one of five BBS-like peptides **(b)** given at 55-minute intervals. At the end of the experiment, a 5 minute pulse of 100 nM sGnRH was administered as a control. Black bars represent application of peptide. The effects of BBS and BBS-like peptides on total GtH-II release when expressed as the mean total hormone released per pulse are summarized in **(c)** and **(d)**, respectively (n = 4 columns/dose; mean hormone basal levels prior to peptide challenge, GtH-II 4.0 ng/mL).

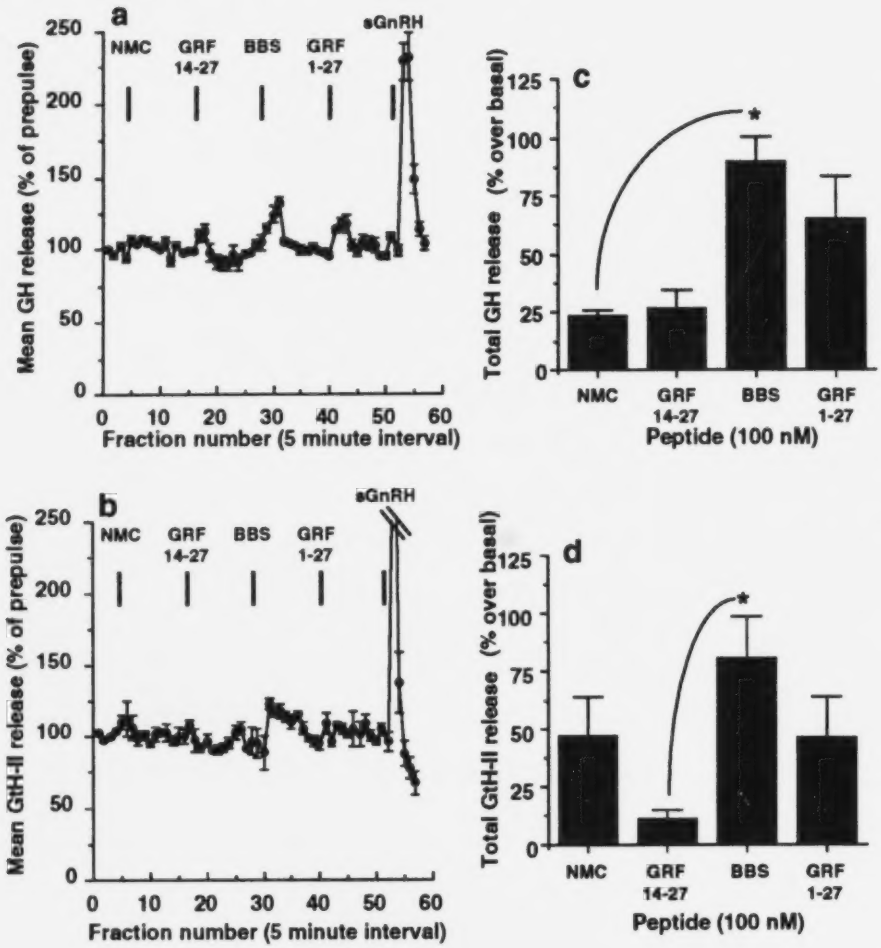

Fig. 4-9. Release profiles of GH (a) and GtH-II (b) in perifusion columns following challenge with BBS-like peptides. Pituitary fragments from gonadal late recrudescent goldfish were exposed to five 5 minute pulses of 100 nM of one of five BBS-like peptides given at 55-minute intervals. At the end of the experiment, a 5 minute pulse of 100 nM sGnRH was administered as a control. Black bars represent application of peptide. The effects of BBS-like peptides on total GH release when expressed as the mean total GH and GtH-II released per pulse are summarized in **(c)** and **(d)**, respectively (n = 4 columns/dose; mean hormone basal levels prior to peptide challenge, GH 7.0 ng/mL; GtH-II 4.4 ng/mL). Asterisk represents significance between groups with Student's t-test, $p < 0.05$.

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Chapter 5

CCK/Gastrin-Like Immunoreactivity in Brain and Gut, and CCK Suppression of Feeding in Goldfish[1]

5.1 Introduction

Cholecystokinin (CCK), gastrin, and the amphibian skin peptide caerulein, belong to a family of peptides characterized by sharing an identical biologically active carboxyl terminal pentapeptide sequence (Dockray, 1976; Vanderhaeghen *et al.*, 1980; Chapter 1). Members of the CCK/gastrin family of peptides are known to be present throughout the vertebrates (Dockray, 1976; Holmquist *et al.*, 1979; Crim and Vigna, 1983; Dockray and Dimaline, 1984), and it has been proposed that discrete CCK and gastrin molecules may have arisen from an ancestral CCK/caerulein-like peptide at the evolutionary level of divergence of amphibians and reptiles (Larson and Rehfeld, 1977; for review Vigna, 1994). In mammals, it is well established that CCK peptides act peripherally at the level of the gut and centrally within specific brain regions to regulate and integrate activities associated with the mechanical digestion of food, and the subsequent psychological and physiological events associated with the onset and termination of feeding (for reviews Baile *et al.*, 1986; Silver and Morley, 1991). The highly conserved biologically active C-terminal sequence of the CCK/gastrin-like peptides throughout vertebrate evolution suggests that actions mediated by these peptides in mammals may also be present in other vertebrates.

In teleosts, CCK-like peptides have been shown to act in parallel fashion to that reported in mammals in altering events associated with the mechanical events of digestion, such as gut motility and gallbladder contraction (Vigna and Gorbman, 1977; Jonsson *et al.*, 1987; Rajjo *et al.*, 1988). The presence of CCK/gastrin-like IR material in endocrine cells and nerve fibres of the gastrointestinal tract of several fish species also

[1] A version of this chapter has been accepted for publication. Himick, BA and Peter, RE. 1994. American Journal of Physiology: Regulatory, Integrative and Comparative Physiology.

supports a role for these peptides in the peripheral regulation of feeding activities (for review Bjenning and Holmgren, 1988). The acute effects of CCK/gastrin-like peptides on the central regulation of feeding behavior in fish however, remain unknown. A widespread distribution of CCK/gastrin-like IR material has been reported within the central nervous system, including the hypothalamic feeding center of the fish brain, indicating a role for CCK/gastrin-like peptides in brain regulation of physiological processes. For example, Notenboom *et al.* (1981) reported CCK/gastrin-like IR perikarya and IR nerve fibres in the caudal hypothalamus of the rainbow trout, *Oncorhynchus mykiss*, while in the green molly *Poecilia latipinna*, fine CCK/gastrin-like IR fibres were reported within regions of the rostral and caudal telencephalon (Batten *et al.*, 1986; Chapter 1). In the sea bass *Dicentrarchus labrax*, CCK/gastrin-like IR fibres and IR cell bodies have also been demonstrated in the ventral telencephalon, preoptic region and caudal hypothalamus (Moons *et al.*, 1992). In these same brain regions, specific binding sites for [^{3}H] -CCK have been described (Moons *et al.*, 1992), suggesting that target sites of action for CCK-like peptides exist within the teleost brain.

Experiments in this chapter investigated the presence of CCK/gastrin-like IR peptides in the goldfish brain and examined the role of CCK/gastrin-like peptides in the peripheral and central regulation of feeding behavior in fish. Since CCK-like peptides are highly effective in releasing both GH and GtH-II *in vitro* from perifused goldfish pituitary fragments (Himick *et al.*, 1993, Chapter 6), the effects of CCK peptide administration on circulating levels of GH and GtH-II were also reported. Data in this chapter provide evidence that CCK/gastrin-like IR is localized within the brain feeding center of fish and that CCK-like peptides suppress feeding behavior when administered centrally or peripherally.

5.2 Materials and Methods

Experimental Animals

Male and female goldfish (25-45 g) (Ozark Fisheries, Stoutland, MO) were maintained for approximately two months under simulated Edmonton natural photoperiod in 1.8 kL tanks which received a constant flow of aerated and dechlorinated city water at 17^{o}C. Fish were fed *ad libitum* twice daily with commercially prepared fish pellets until either transfer to experimental observation aquaria, where fish were further acclimated for

two weeks, or were used for immunohistochemical studies.

Immunohistochemistry of CCK/Gastrin-Like Peptides in Goldfish Brain and Gut

Brains of anesthetized goldfish were perfused *in situ* via the bulbous arteriosus with ice-cold 0.1 M phosphate buffered saline (PBS; pH 7.4), followed by 4% paraformaldehyde containing 0.2% picric acid in PBS, pH 7.4. Brain tissues, as well as tissue segments from the anterior gastrointestinal tract, were removed, post-fixed, cryoprotected and sectioned following standard procedures described in Chapter 4.

To block non-specific binding of the CCK/gastrin antisera, tissue sections were initially incubated with 30% normal goat serum (Calbiochem, La Jolla, CA). This was followed by incubation with CCK/gastrin antisera G03 (1:300) for 18 to 20 hours at 4°C. Sections were subsequently exposed to goat anti-rabbit immunoglobulin G, which was conjugated to tetramethylrhodamine isothiocyanate (TRITC; 1:100, Sigma, St. Louis, MO), for 60 minutes in the dark. Sections were mounted using carbonate-buffered glycerol/p-phenylenediamine (pH 8.5) and immunoreactive staining was photographed using a Zeiss fluorescence microscope. For use of avidin-biotin coupled with peroxidase as an immunoreactive label, endogenous peroxidase activity was intitally inactivated by incubating sections in methanol containing 0.3% hydrogen peroxide for 30 minutes. Following incubation in normal goat serum and exposure to CCK/gastrin antisera G03 antisera as outlined above, IR was examined using standard avidin-biotin immunoperoxidase procedures (ABC VectaStain Kit, Vector Labs, Burlingame, CA). Immunoreactivity was visualized as a brown reaction product following incubation with the chromagen diaminobenzidine (Sigma) in 0.05 M TRIS buffer (pH 7.6) containing 0.01% hydrogen peroxide. Immunoreactive staining was photographed using a Ziess light microscope.

Antisera and Specificity Controls

A description of G03 rabbit anti-CCK/gastrin, kindly donated by Dr. A.C. Jonsson (University of Goteborg, Sweden), has been documented by radioimmunoassay (RIA) previously (Jonsson *et al.*, 1987). Briefly, CCK 4 (CCK_{30-33}) was conjugated to

bovine serum albumin with carbodiimide, and injected into rabbits. The specificity of the resulting G03 antiserum was then examined by conducting displacement curves for several CCK/gastrin-like peptides; CCK4, gastrin 5, pentagastrin, CCK6, CCK7, CCK8-s, CCK8-ns, and caerulein all showed parallel displacement with ID_{50} values ranging from 0.9 to 20.4 pmol/litre. Bombesin, litorin, and gastrin-releasing peptide did not bind to the antibody. In addition, routine tests for the specificity of the immunoreactive staining were performed. Staining by G03 was totally quenched when preabsorbed for 24 hours with CCK8-s (10 nmol/mL diluted antisera) or gastrin 17-s (10 nmol/mL), but not when preabsorbed with bombesin (100 nmol/mL). Staining was abolished following the omission of primary antisera and replacement with normal rabbit serum, or following omission of secondary antisera. To identify specific brain nuclei, serial sections were stained with cresyl violet.

5.2.2 Observational Experiments

To examine the effects of CCK/gastrin-like peptides on feeding behavior, fish were anesthetized with 0.05% MS222, weighed to the nearest 0.1 g and randomly assigned to 65 L observational aquaria containing flow-through, aerated water at 17 - 19 ^{o}C (3 fish per aquarium: photoperiod 16L : 8D). Fish were identified by individual markings. All test aquaria contained gravel substrate and floating synthetic weeds, while tank sides were covered by opaque barriers to minimize external disturbances. Following the standardized protocol developed in Chapter 2, goldfish were fed once daily a 2% bw ration of floating pellets.

Observational experiments were conducted in female and male fish in gonadal regressed conditions over the light phase of 09:30 h to 16:30 h (refer to Chapter 2). On experimental day, individual floating pellets consumed by each fish were recorded over a 30 minute interval by direct observation, commencing at 15 minutes after fish received either a peptide or saline (control) injection. At the end of the 30 minute interval, fish were netted and anesthetized and, when fin and body movements ceased, a 500 µL blood sample was collected from the caudal vasculature using a 25-gauge, 5/8 inch needle attached to a 1 mL syringe. After clotting for approximately 3-4 hours at 4^{o}C, samples were centrifuged, and collected serum was stored at -20^{o}C until analysed for GH and GtH-II levels.

For intraperitoneal (ip) injection experiments, fish were netted, anesthetized, and a 3 μL/g wet body weight volume of either saline (0.9% NaCl) or peptide was administered just caudal to the pelvic fins using a 26-gauge, 5/8 inch needle attached to a Hamilton syringe (250 μL; Fisher Scientific). Following injection, fish were returned to their respective tanks and allowed to recover, which normally did not exceed one or two minutes.

Ventricular brain injections (icv) were administered following routine procedures outlined in the stereotaxic atlas for the goldfish forebrain (Peter and Gill, 1975) and in Chapter 3. Briefly, a bone flap was cut in the roof of the skull and was folded back to expose the brain. An injection needle was then placed stereotaxically in the brain ventricle and, following injection of either goldfish physiological saline or peptide (2 μL), the needle was withdrawn and the skull flap repositioned. Fish were returned to their respective tanks and normally recovered from anesthesia within one or two minutes. Icv injections were administered at brain co-ordinates of +1.3 mm (anterior to posterior commissure), midline at a depth of +2.0 mm, resulting in an approximate placement of the needle in the brain ventricle in the region of the NPO and nucleus preopticus periventricularis (NPP).

Peptides administered

Sulfated CCK8 (CCK8-s) was purchased from Richelieu Biotechnologies (Saint-Hyacinthe, QC). Sulfated gastrin 17 (G17-s) was purchased from Sigma. Nonsulfated CCK8 (CCK8-ns) was purchased from Pennisula Laboratories, Inc. (Belmont, CA). Bombesin-14 (used for examination of antisera specificity) was purchased from Bachem California (Torrance, CA).

Hormone Measurements and Data Analyses

Concentrations of GH and GtH-II in serum were determined by specific RIA using purified common carp GH (Marchant *et al.*, 1989) or common carp GtH-II (Peter *et al.*, 1984; Van Der Kraak *et al.*, 1992) as both standards and radioligands (refer to Chapter 2 for protocol description). Differences in serum hormone levels or the amount of food consumed between control and experimental fish were analysed using either one way

ANOVA followed by Duncan's multiple range test, or by Student's t-test. For all experiments, significance was considered at $p \leq 0.05$ and error bars are representative of standard error of measurement (SEM).

5.3 Results

5.3.1 CCK/Gastrin-Like IR in the Goldfish Brain and Gut

5.3.1.1 Forebrain

Telencephalon

Within ventral regions of the goldfish telencephalon, sparsely distributed, thin beaded CCK/gastrin-like IR fibres were present in the area ventralis telencephali pars ventralis (Vv), area ventralis telencephali pars dorsalis (Vd), and area ventralis telencephali pars lateralis (Vl). IR fibres and several smaller CCK/gastrin-like IR perikarya were observed in the area ventralis telencephali pars supracommissurali (Vs) (Fig. 5-1 A and B). Only a few, sparsely distributed CCK/gastrin-IR fibres were detected within more dorsal regions of the telencephalon.

Diencephalon

CCK/gastrin-like IR fibres increased in density within the preoptic region of the ventral anterior hypothalamus at the level of the nucleus entopeduncularis (NE) and nucleus preopticus periventricularis (NPP) (Fig. 5-1 B). Occasionally, a few small CCK/gastrin-like IR perikarya were observed in the NPP. Within the nucleus preopticus (NPO), thin-beaded IR-fibres appeared to run amongst both the magnocellular cells and the smaller periventricular cells; a few CCK/gastrin-like IR cell bodies were also detected in the NPO (Fig. 5-1 C and D).

Throughout all goldfish brain regions examined, the greatest concentration of CCK/gastrin-like IR material was localized in the more ventro-caudal regions of the hypothalamus. The nucleus lateral tuberis pars anterioris (NLTa), nucleus lateral tuberis pars posterioris (NLTp), and nucleus anterior tuberis (NAT) contained dense staining IR-fibres; CCK/gastrin-like IR perikarya were also detected in the NLTp (Fig. 5-1 D and E). Within the ventro-posterior and inferior lobes of the hypothalamus, fibre tracts containing CCK/gastrin-like IR were localized in the nucleus recessus lateralis (NRL), nucleus glomerulosus (NG), nucleus preglomerulosus (NPGl), nucleus diffusus tori lateralis

(NDTL), and nucleus diffusus lobi inferioris (NDLI) (Fig. 5-1 E, F; Fig. 5-2 b, c, f). These dense staining fibres appeared to originate from CCK/gastrin -like IR perikarya located periventricularly within the NLTp, nucleus posterioris periventricularis (NPPv), NRL, and nucleus recessus posterioris (NRP) (Fig. 5-1 E, F and G; Fig. 5-2 g; Fig. 5-3 b, c).

Several more dorsally located regions of the goldfish diencephalon exhibited concentrated CCK/gastrin-like IR fibres and perikarya, including the nucleus habenularis (NH) (Fig. 5-1 E; Fig. 5-2 e) and the nucleus dorsomedialis thalami (NDM), nucleus dorsolateralis thalami (NDL), and nucleus ventromedialis thalami (NVM) of the thalamus (Fig. 5-1 E and F). Fine-beaded CCK/gastrin-like IR fibres were also detected throughout the optic tectum (OTec), with a greater density of IR staining contained in the inner and outer layers (Fig. 5-1 G; Fig. 5-2 h). Occasionally, CCK/gastrin-like IR cell bodies were detected in the periphery of the OTec.

5.3.1.2 Midbrain and Hindbrain

Within the midbrain tegmentum (MT), a nerve fibre tract containing CCK/gastrin-like IR was observed which, in the more anterior MT, was detected laterally but then appeared to be located more centrally at the level of the caudal MT (Fig. 5-1 G). In the anterior MT, this CCK/gastrin-like IR fibre tract appeared to enter the hypothalamic inferior lobe laterally at the level of the NG and NPGl. Extensive CCK/gastrin-like IR staining was also detected within the nucleus interpeduncularis (NIp) of the midbrain (Fig. 5-3 d).

In addition, fibres containing CCK/gastrin-like IR were detected within several areas of the hindbrain, including the vagal lobe (VL) (Fig. 5-1 H; Fig. 5-3 e, f), and to a lesser extent the facial lobe (FL) (Fig. 5-1 H). Within the VL, a band of CCK/gastrin-like IR fibres was observed peripherally in the outer lateral layer. Additionally, IR fibres were concentrated in the ventral VL (Fig. 5-3 f). CCK/gastrin-like IR perikarya were detected in the VL (Fig. 5-3 e) and occasionally also within the ventral FL.

5.3.1.3 Gastrointestinal Tract

Only the anterior approximately one-third segment of the goldfish gastrointestinal

tract was examined. However, CCK/gastrin-like IR endocrine cells were found scattered throughout the mucosal layer (Fig. 5-3 h i and ii). In addition, nerve fibres containing CCK/gastrin-like IR were detected in the submucosal and circular muscle layers.

5.3.2 CCK-Like Peptides and Feeding Behavior in Goldfish

5.3.2.1 Acute effects of peripheral administration of CCK8-s At 30 minutes following administration of food (45 minutes post-injection), goldfish (n = 7-10 / treatment) receiving an ip injection of either 50 or 500 ng/g CCK8-s exhibited a significant suppression in feeding activity relative to saline-injected controls (Fig. 5-4 a). Fish receiving 50 ng/g CCK8-s decreased their food intake to 54% of that consumed by controls, while administration of 500 ng/g CCK8-s resulted in a 81% decrease in food intake relative to food consumed by saline-injected fish. Serum GH and GtH-II levels remained unchanged following injection of both doses of CCK8-s relative to control fish (Fig. 5-4 b and c).

5.3.2.2 Acute effects of peripheral administration of CCK8-s and CCK8-ns At 30 minutes following administration of food (45 minutes post-injection), goldfish (n = 7-10 / treatment) receiving an ip injection of only CCK8-s (100 ng/g) exhibited a significant suppression in food intake relative to fish receiving either an equimolar concentration of CCK8-ns (95.6 ng/g) or saline (controls). Relative to controls, fish receiving CCK8-s exhibited a 58% decrease in the amount of food consumed while fish receiving CCK8-ns reduced their food intake only by 11% (Fig. 5-5 a). Serum GH levels were elevated only in fish receiving CCK8-s, when compared to levels of saline-injected fish (Fig. 5-5 b). Serum GtH-II levels remained unchanged following injection of CCK8-s, CCK8-ns, or saline (Fig. 5-5 c).

5.3.2.3 Acute effects of icv administration of CCK8-s At 30 minutes following administration of food (45 minutes post-injection), goldfish (n = 8-9 / treatment) which received an icv injection of 50 ng/g CCK8-s exhibited a 95% decrease in food intake relative to the amount consumed by saline-injected fish (Fig. 5-6 a). Additionally, icv injection of CCK8-s resulted in an approximate two-fold increase in serum GH levels relative to saline -injected fish, while GtH-II remained unchanged between the two

groups (Fig. 5-6 b and c).

In a second experiment, at 30 minutes following administraton of food (45 minutes post-injection), goldfish (n = 8-9/ treatment) which received an icv injection of 5 ng/g CCK8-s exhibited a 50% decrease in food intake relative to levels consumed by control fish (Fig. 5-7 a). Serum GH levels remained unaltered following injection of CCK8-s (Fig. 5-7 b).

5.4 Discussion

5.4.1 Distribution of CCK/Gastrin-Like IR in Goldfish Brain and Gut

These studies indicate that in the goldfish, CCK/gastrin-like IR fibres and IR perikarya are widely distributed in the forebrain, midbrain, and hindbrain. A highly concentrated and extensive CCK/gastrin-like IR fibre and IR perikarya system is prominent within the ventral goldfish hypothalamus, and, in particular, in regions within the inferior lobe of the hypothalamus. Recently, we described an extensive CCK/gastrin-like IR fibre system in the PPD of the goldfish anterior pituitary with a high density of IR fibres distributed amongst both the somatotropes and gonadotropes (Himick *et al.*, 1993; Chapter 6). On the basis of the present study, and of our previous report (Himick *et al.*, 1993), CCK/gastrin-like IR fibres appear to originate in the NLT of the goldfish hypothalamus, course into the hypophysial stalk, and then enter the PPD of the anterior pituitary.

The localization of CCK/gastrin-like IR fibres and IR perikarya within specific regions of the goldfish brain confirms and extends earlier studies on other teleosts (refer to Introduction). CCK/gastrin-like IR material was also localized peripherally within the goldfish gastrointestinal tract. In the present study, only regions of the anterior one-third portion of the gut (goldfish lack a stomach) were examined for CCK/gastrin-like IR material. Both endocrine cells of the mucosa and nerve fibres of the submucosa and circular muscle layers were found to contain CCK/gastrin-like IR material. These findings are also supported by studies in other fish species. For example, CCK/gastrin-like IR nerve fibres have been described within the myenteric plexus of the cardiac stomach, and in the circular muscle layer and submucosa layer of the stomach in the cod, *G. morhua* (Jonsson *et al.*, 1987). Nerve fibres containing CCK/gastrin-like IR

material have also been reported in the intestine of both the common carp *Cyprinus carpio* (Bjenning and Holmgren, 1988) and the ide, *Leuciscus idus* (Langer *et al.*, 1979), the rectum of *Poecilia reticulata* (Langer *et al.*, 1979) and in the stomach and intestine of the sea scorpion, *Myoxocephalus scorpius* (Reinecke, 1981). Endocrine cells containing CCK/gastrin-like IR have been documented in the mucosal layers of the stomach (Jonsson *et al.*, 1987) and the intestine (Langer *et al.*, 1979; Noaillac-Depeyre and Hollande, 1981; Holmgren *et al.*, 1982) of several fish species.

5.4.2 CCK Acts to Alter Feeding Behavior in the Goldfish

Early anatomical studies (Herrick, 1905; Crosby and Showers, 1969; Finger, 1978) and research involving electrical stimulation of specific areas of the teleost brain (Grimm, 1960; Demski and Knigge, 1971; Savage, 1971; Demski, 1973) have demonstrated a "hypothalamic feeding area" (HFA) in the hypothalamic inferior lobes (for review Demski, 1981). More specifically, electrical stimulation of the lateral recess of the third ventricle (NRL) in the bluegill sunfish, *Lepomis macrochirus*, and the tilapia (*Tilapia macrocephala*) have induced searching behaviors, snapping at food and snapping up gravel (Demski and Knigge, 1971; Demski, 1973). In the goldfish, electrical stimulation in areas just dorsal to the NRL and below the NPGl have also produced an increase in feeding activity (Savage and Roberts, 1975). Likewise, altered feeding responses in the common carp following inferior lobe stimulation have been reported (Redgate, 1974). Reduced food intake in goldfish following hypothalamic bilateral lesioning within the lateral or inferior hypothalamic lobes have also been described (Roberts and Savage, 1978).

In the present study, CCK/gastrin-like IR material was localized in greatest density to more ventro-caudal regions of the hypothalamus, part of the proposed HFA. Areas such as the NRL, NPGl, and NDLI which, when electrically stimulated, have produced alterations in feeding activity in goldfish and other teleosts (see above), were also found to contain a high density of CCK/gastrin-like IR material. These results suggest that CCK-like peptides are involved in the regulation of feeding behavior in the goldfish. To examine this possibility, goldfish received a single injection of CCK8-s either peripherally or centrally into the brain ventricular system. Data presented here indicate that the sulfated form of CCK8 is effective in suppressing the amount of food consumed

by fish during the 30 minute feeding period, beginning at 15 minutes after injection of the peptide by either route. When peripherally administered, a dose of 50 ng/g CCK8-s caused a 54% suppression of food intake whereas 500 ng/g CCK8-s caused an 81% suppression relative to controls. Peripheral injection of the nonsulfated form of CCK8 did not cause a significant suppression in the amount of food consumed relative to control fish, compared to the 58% reduction in food intake found in fish following injection of an equimolar dose of CCK8-s. These findings support other studies in teleosts which indicate that the CCK/gastrin receptor requires the presence of a sulfated tyrosine for enhanced activation (Vigna and Gorbman, 1977; Holstein, 1982; Aldman and Holmgren, 1987; Rajjo *et al.*, 1988; Himick *et al.*, 1993; Vigna, 1994).

While it remains unknown where CCK acts in the goldfish to suppress feeding behavior when administered peripherally, it is possible that CCK exerts its effects, in part, through alterations in gut contractility and/or emptying, either of which may subsequently result in neural feedback to the brain. In several fish species, CCK has been shown to alter stomach muscle contractility *in vitro* (Jonsson *et al.*, 1987), and increase the contraction of gallbladder muscle strips *in vitro* (Vigna and Gorbman, 1977; Aldman and Holmgren, 1987; Rajjo *et al.*, 1988). Additionally, CCK may also act in parallel fashion to that observed in mammals to alter the tonicity of peripheral vagal input to the hindbrain (for reviews Morley, 1987; Silver and Morley, 1991). The localization of CCK/gastrin-like IR in the goldfish vagal lobes in the present study, as well as the presence of specific bindings sites for [^{3}H]-CCK in the teleost hindbrain (Moons *et al.*, 1992) would support such CCK-mediated peripheral actions.

Administration of CCK8-s into the third brain ventricle of the goldfish also resulted in a suppression of feeding behavior. A high dose of 50 ng/g CCK8-s injected icv resulted in a 95% decrease in the amount of food consumed. When a ten-fold lower dose of CCK8-s (5 ng/g) was administered icv, food intake was suppressed to 50% relative to feeding levels observed in control fish. As with peripherally administered CCK8-s, it remains unknown where in the goldfish brain CCK8-s acts to mediate suppressed feeding when centrally injected. Specific binding sites for CCK-like peptides have been documented throughout regions of the forebrain, midbrain, and hindbrain of the sea bass, in areas which closely correspond to brain regions containing CCK/gastrin-like IR staining in both the sea bass (Moons *et al.*, 1992), and the goldfish. In addition, cell

bodies containing CCK/gastrin-like IR were localized in periventricular regions of the goldfish NRL, NPPv, and NRP, and it remains probable that injection of CCK8-s into the ventricular system also had direct contact with these areas. One other possibility is that the high dose of CCK injected icv may have resulted in some leakage of the peptide to the periphery introducing peripherally-mediated CCK actions.

Several areas outside of the hypothalamic inferior lobe region also contained a high density of CCK/gastrin-like IR material, such as the NH of the epithalamus and the NIp of the midbrain. In teleosts, the paired NH receives fibers from various regions of the forebrain; afferent fibres are received from cells of the preoptic recess, as well as from the paraolfactory segment (Ariens Kappers *et al.*, 1965). Additionally, in some fish species, fibres also arise from the pineal gland to innervate the NH (Holmgren, 1920). It remains possible that CCK-like peptides can regulate feeding activity based on such environmental stimuli as olfaction (forebrain input), and photoperiod (pineal gland input), both of which are known to influence the amount of food consumed in goldfish (Rozin and Mayer, 1964; Stacey and Kyle, 1983). From the NH, anatomical studies have described a pathway of efferent fibers running to the thalamic region and MT, with fibres eventually terminating in the NIp (Holmgren, 1920). These regions were all areas which were found to contain moderate to dense amounts of CCK/gastrin-like IR material.

While the specificity of CCK-induced feeding suppressive effects in goldfish are presently being examined in our laboratory through investigation of CCK analogues, several behavioral actions were displayed by the fish following peptide injection indicating that decreased food intake by CCK8-s was likely not due to the production of malaise. In goldfish, malaise usually results in characteristic behavioral traits which include lowering of the dorsal fins, retreat to aquatic vegetation within the aquarium, and decreased locomotor activity. During experiments, CCK and saline-injected fish were present within the same tank and when presented with a food source, both CCK-injected and control fish responded by increasing locomotor activity and actively searching for the food source. However, unlike saline-injected controls, fish injected either ip or icv with CCK consumed a reduced amount of food. Furthermore, goldfish are area copy feeders; the location of a food source by one fish signals other fish to its presence and as a result, group feeding occurs (Pitcher, 1986). The fact that saline -injected fish were consuming

food in the presence of CCK-injected fish indicates the effectiveness of CCK in its suppressive actions on feeding behavior. While the suppressive actions of ip or icv-injected bombesin on food intake have recently been reported in the goldfish (Himick and Peter, 1994; Chapter 6), data presented in this study demonstrate for the first time that a member of the CCK/gastrin family of peptides is capable of acutely decreasing feeding behavior in fish following administration either peripherally or centrally into the third brain ventricle.

Associated with injection of CCK8-s and reduced food intake were inconsistent alterations in circulating levels of serum GH. In two experiments, elevations in serum GH accompanied CCK-mediated feeding suppression, while in other experiments no changes were observed in serum GH. Initially we had speculated that the increase in serum GH levels following ip or icv injection of CCK8-s may have been the result of a direct action of CCK8-s on the somatotropes. Recently, we reported that CCK8-s acts at the level of the goldfish pituitary to stimulate GH release (Himick *et al.*, 1993). However, concomitant with CCK8-s -mediated GH secretion from pituitary fragments *in vitro* was the release of GtH-II (Himick *et al.*, 1993). Since no changes in circulating serum GtH-II levels were observed in the present studies, it seems unlikely that injection of CCK8-s resulted in an increase in serum GH through direct actions at the level of the pituitary. Alternatively, CCK8-s may have stimulated GH release via interaction(s) with other known physiological regulators of GH release. It is established that in the goldfish, brain levels of IR -somatostatin (SRIF) (Marchant *et al.*, 1989) and salmon gonadotropin-releasing hormone (GnRH) (*Yu et al.*, 1991) fluctuate according to gonadal sexual stage. While goldfish in this study were in gonadal regression, the experiments were conducted at different times of the year (June to December) and differential GH responsiveness may occur to CCK if SRIF or GnRH are undergoing seasonal changes in their secretion patterns.

In mammalian studies, inconsistent effects on GH and GtH levels following CCK injection have similarly been reported. In rats, CCK is capable of altering GH and luteinizing hormone levels within 15 and 5 minutes of third ventricular injection respectively, but has no effect when administered intravenously at physiological doses (Vijayan *et al.*, 1979). In humans, intravenous admininstration of CCK8 has been shown to have either no effect on basal GH secretion (Nair *et al.*, 1986), or a dose-

dependent increase in GH levels (Calogero *et al.*, 1993). Despite these inconsistent findings, it is established that CCK8-s has a direct stimulatory effect on GH and GtH secretion at the level of the mammalian pituitary *in vitro* (Vijayan *et al.*, 1979; Morley *et al.*, 1979; Matsumura *et al.*, 1984).

In summary, these studies demonstrate that CCK/gastrin-like IR nerve fibres and IR perikarya are widely distributed throughout regions of the goldfish forebrain, midbrain, and hindbrain, as well as within nerve fibres and endocrine cells of the goldfish gastrointestinal tract. The localization of CCK/gastrin-like IR in areas associated with feeding activity, including the hypothalamic inferior lobes and in the gut, strongly suggests that CCK-like peptides are involved in the regulation of food intake in the goldfish. This is confirmed by the demonstration of a suppressive effect of CCK8-s on feeding behavior in goldfish following either peripheral or third brain ventricle injection. Despite the fact that CCK8-s is capable of directly regulating anterior pituitary GH and GtH-II release *in vitro*, inconsistent changes in circulating hormone levels following *in vivo* injection of CCK8-s concomitant with reduced food intake indicate that alterations in GH and GtH-II are not necessary for CCK to mediate its effects of suppressed feeding in fish. Studies in this paper indicate that CCK-like peptides likely play a physiological role in the short-term regulation of feeding behavior in teleosts.

Fig. 5-1 (A-H). Distribution of CCK/gastrin-like IR in the goldfish forebrain, midbrain and hindbrain. Fine dots represent CCK/gastrin-like IR nerve fibre terminals; larger dark circles represents CCK/gastrin-like IR perikarya. Density of fine dots and dark circles indicate the abundance of CCK/gastrin-like IR material within specific brain regions. Intervals between successive sections are A-B: 800 μm; B-C: 800 μm; C-D: 800 μm: D-E: 3000 μm.

List of abbreviations are as follows: AP (area pretectalis); C (cerebellum); CM (corpus mamillare); Dc (area dorsalis telencephali pars centralis); Dd (area dorsalis telencephali pars dorsalis); Dl (area dorsalis telencephali pars lateralis); Dl_v (area dorsalis telencephali pars lateralis ventralis); Dm (area dorsalis telencephali pars medialis); FL (facial lobe); HOC (horizontal commissure); NAP_v (nucleus anterioris periventricularis); MT (midbrain tegmentum); NAH (nucleus anterioris hypothalami); NAT (nucleus anterior tuberis); NCH (nucleus cerebellosus hypothalami); NDL (nucleus dorsolateralis thalami); NDLI (nucleus diffusus lobi inferioris); NDM (nucleus dorsomedialis thalami); NDTL (nucleus diffusus tori lateralis); NE (nucleus entopeduncularis); NG (nucleus glomerulosus); NH (nucleus habenularis); NLT (nucleus lateral tuberis); NLT_l (nucleus lateral tuberis pars lateralis); NLT_p (nucleus lateral tuberis pars posterioris); NP (nucleus pretectalis); NPG_l (nucleus preglomerulosus pars lateralis); NPG_m (nucleus preglomerulosus pars medialis); NPO (nucleus preopticus); NPP (nucleus preopticus periventricularis); NPP_v (nucleus posterioris periventricularis); NPT (nucleus posterioris tuberis); NRL(nucleus recessus lateralis); NRP (nucleus recessus posterioris); NT (nucleus tenia); NTP (nucleus posterioris thalami); NVM (nucleus ventromedialis thalami); OC (optic chiasma); OT (optic tract); OTec (optic tectum); PC (posterior commissure); PIT (pituitary); SD (saccus dorsalis); Vd (area ventralis telencephali pars dorsalis); Vl (area ventralis telencephali pars lateralis); Vv (area ventralis telencephali pars ventralis); Vs (area ventralis telencephali pars supracommissuralis); VL (vagal lobe); VN (vagal nerve) (nomenclature after Peter and Gill, 1975).

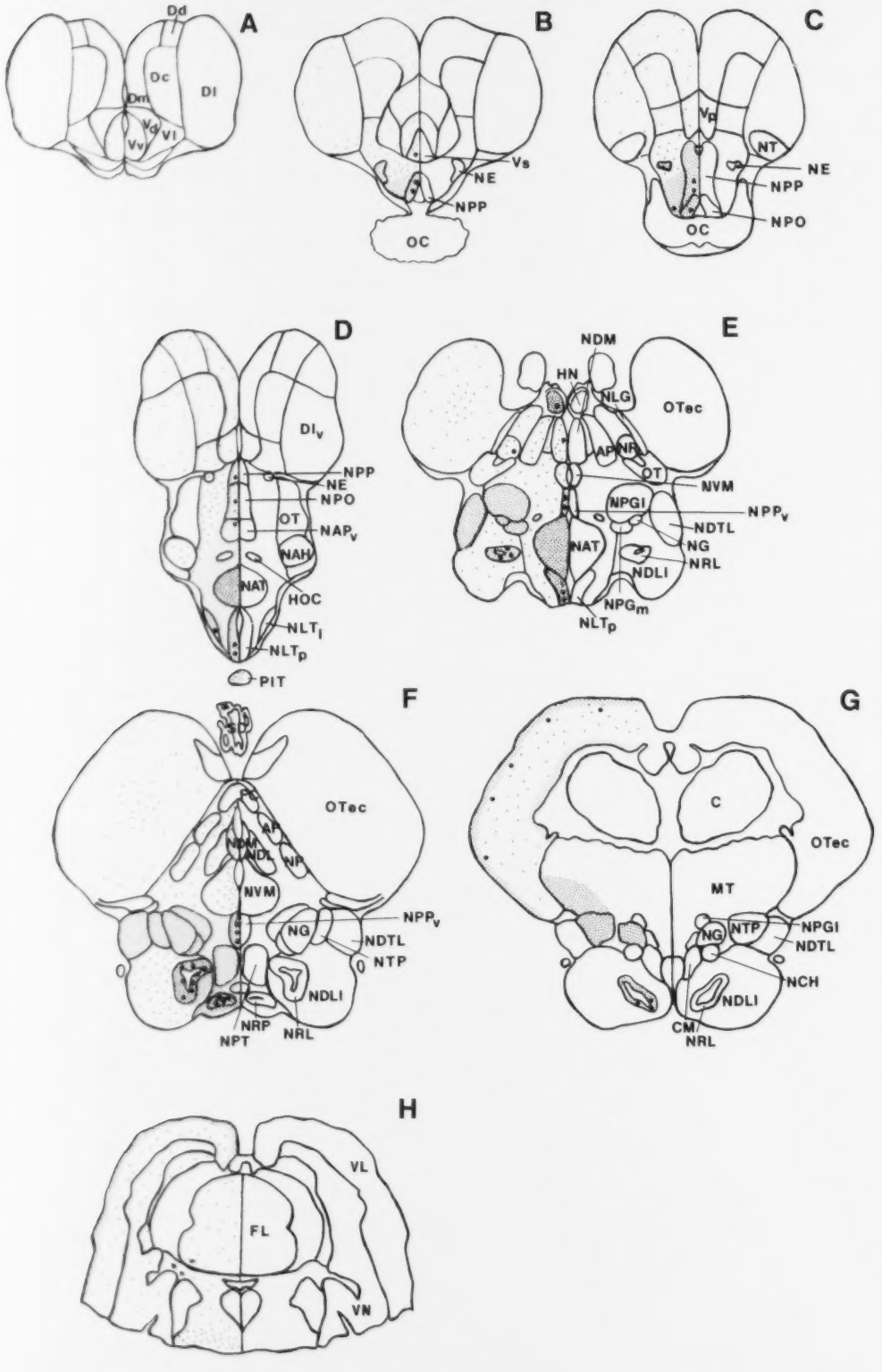
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Fig. 5-2. CCK/gastrin-like IR in the hypothalamic feeding area of the goldfish. **(a)** Anterior inferior lobe of goldfish hypothalamus stained with cresyl violet to indicate nuclear areas of NRL, NPPv, NAT, NLT and NDLI; refer to Fig. 5-1 for list of abbreviations, arrow indicates NRL area where CCK/gastrin-like IR cell bodies are found as shown in (b), v = ventricle; 40X; scale bar = 275 μm; **(b)** Rhodamine-labeled CCK/gastrin-like IR cell bodies (arrow) located periventricularly within the NRL; 400X; scale bar = 30 μm; **(c)** Distribution of CCK/gastrin-like IR fibres and IR perikarya (arrow) within the ventro-posterior hypothalamus using ABC coupled to peroxidase; 40X; scale bar = 250 μm; **(d)** Serial section of inferior lobe of hypothalamus to (c) showing abolished CCK/gastrin-like staining following preabsorption with CCK8-s (control); 40X; scale bar = 250 μm; **(e)** Rhodamine-labeled CCK/gastrin-like IR fibres within the ventral NH; 400X; scale bar = 30 μm; **(f)** Rhodamine-labeled CCK/gastrin-like IR fibres within the NDLI and NDTL regions of the inferior lobe of the hypothalamus; 100X; scale bar = 80 μm; **(g)** Rhodamine-labeled CCK/gastrin-like IR cell bodies localized in the NPPv and in contact with the ventricular system; 400X; scale bar = 30 μm; **(h)** CCK/gastrin-like IR rhodamine-labeled fibres in the outer layer of the OTec (arrow), ns = non-specific staining; 400X; scale bar = 80 μm.

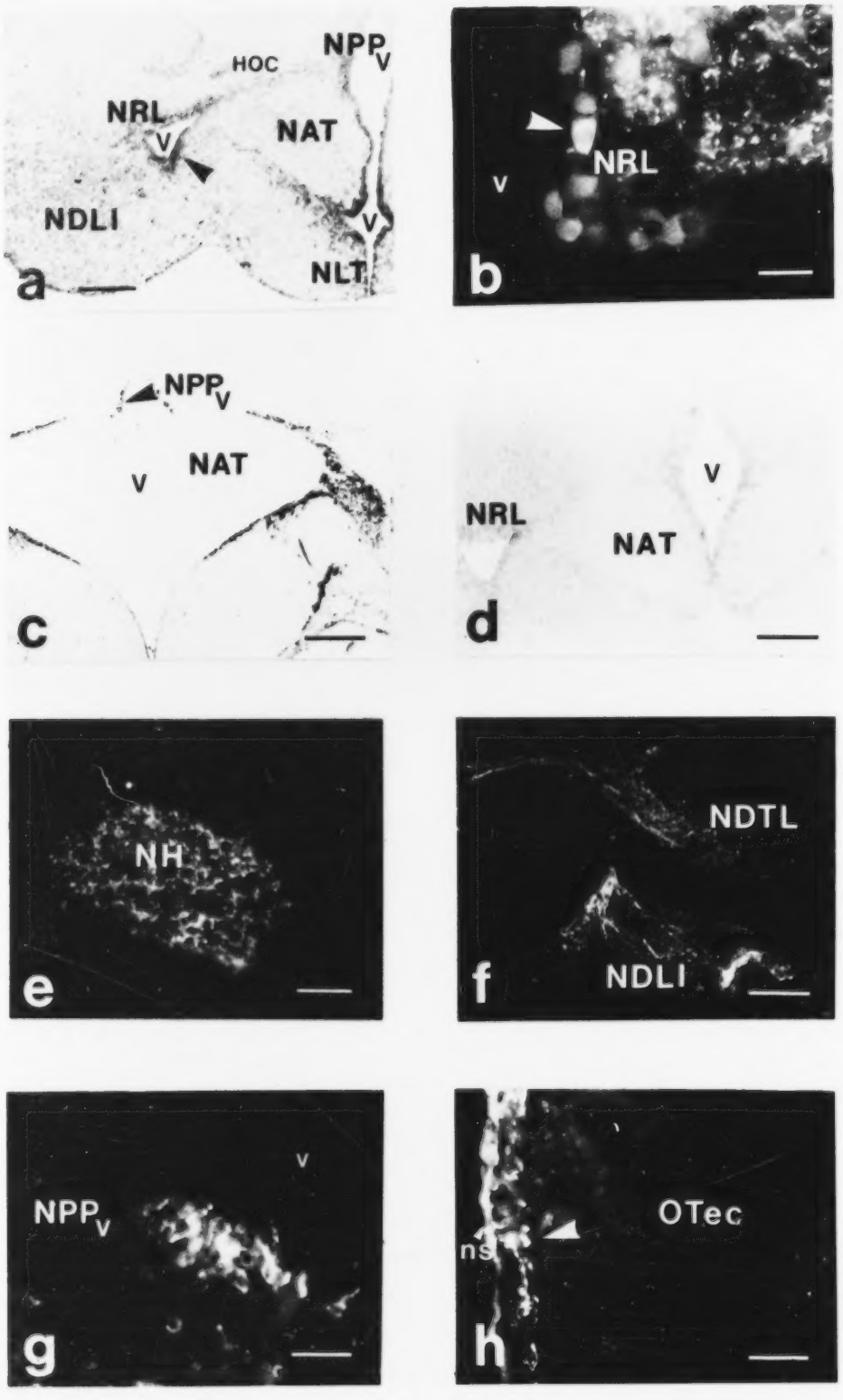
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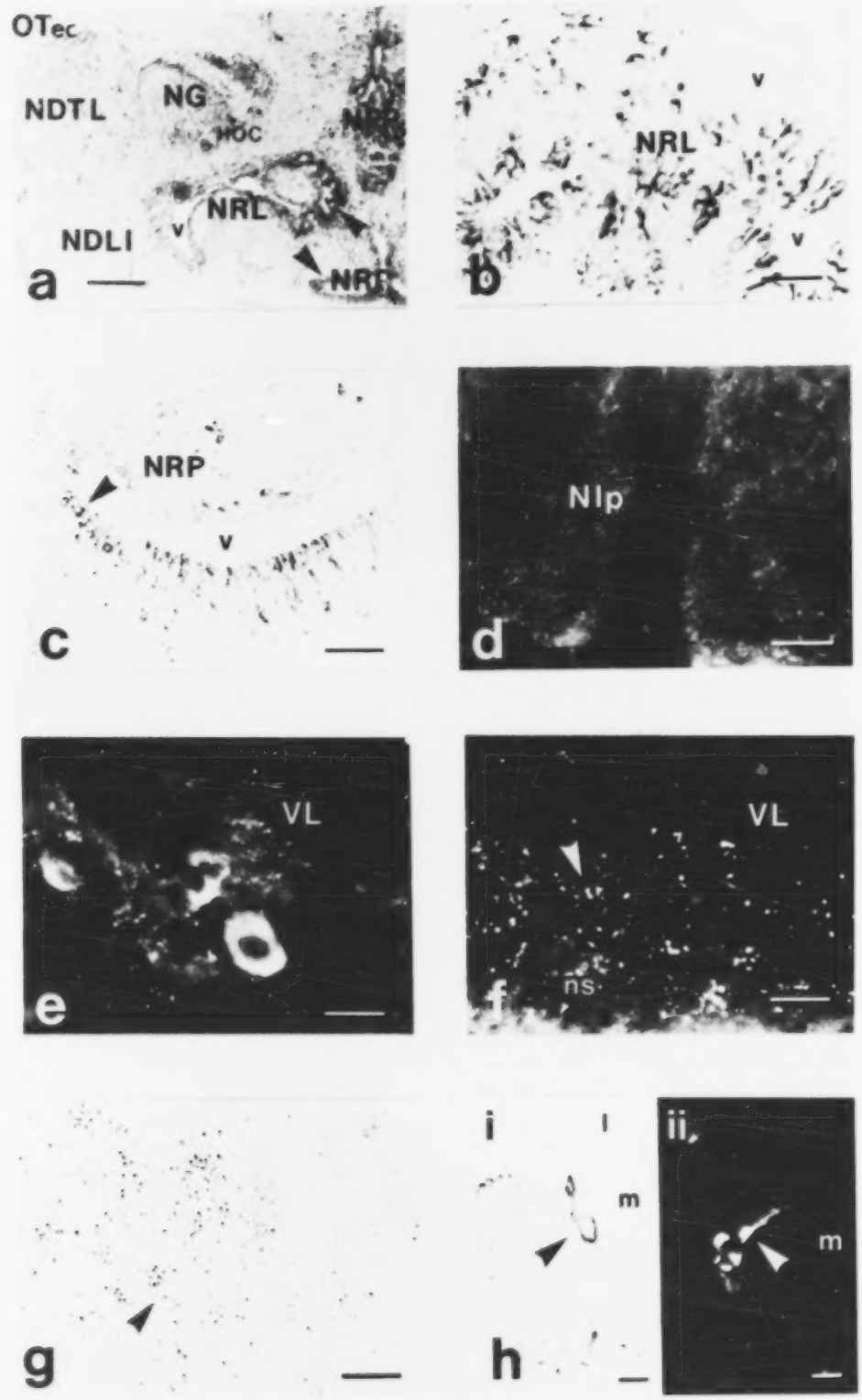
Fig. 5-3. CCK/gastrin-like IR in the ventro-posterior hypothalamus and gut of the goldfish. **(a)** Posterior inferior lobe of goldfish hypothalamus stained with cresyl violet to indicate nuclear areas of NRL, NRP, NPPv, NDLI, NDTL, NG, and OTec; refer to Fig. 5-1 for list of abbreviations; arrows indicate sites of CCK/gastrin-like IR cell bodies localized in serial sections (b) and (c), v = ventricle; 40X; scale bar = 275 μm; **(b)** serial section to (a) identifying periventricular CCK/gastrin-like IR cell bodies within the NRL (ABC coupled to peroxidase); 400X; scale bar = 30 μm; **(c)** serial section to (a) demonstrating periventricularly located CCK/gastrin-like IR perikarya (arrow) and IR nerve fibres within the NRP (ABC coupled to peroxidase); 100X; scale bar = 80 μm; **(d)** Rhodamine-labeled CCK/gastrin-like IR fibres within the NIp of the MT, 400X; scale bar = 30 μm; **(e)** CCK/gastrin-like IR nerve fibres and IR cell bodies labeled with rhodamine within the VL; 400X; scale bar = 30 μm; **(f)** Rhodamine labeled CCK/gastrin-like IR nerve fibres in the ventral region of the VL (arrow), ns = non-specific staining; 400X; scale bar = 30 μm; **(g)** CCK/gastrin-like IR fibres within POA (arrow) (ABC coupled to peroxidase); 400X; scale bar = 30 μm; **(h)** Endocrine cells (arrows) containing CCK/gastrin-like IR within the intestinal mucosa stained with ABC **(i)** and rhodamine **(ii)**, l = lumen of intestine, m = mucosal layer; 400X; scale bar = 10 μm.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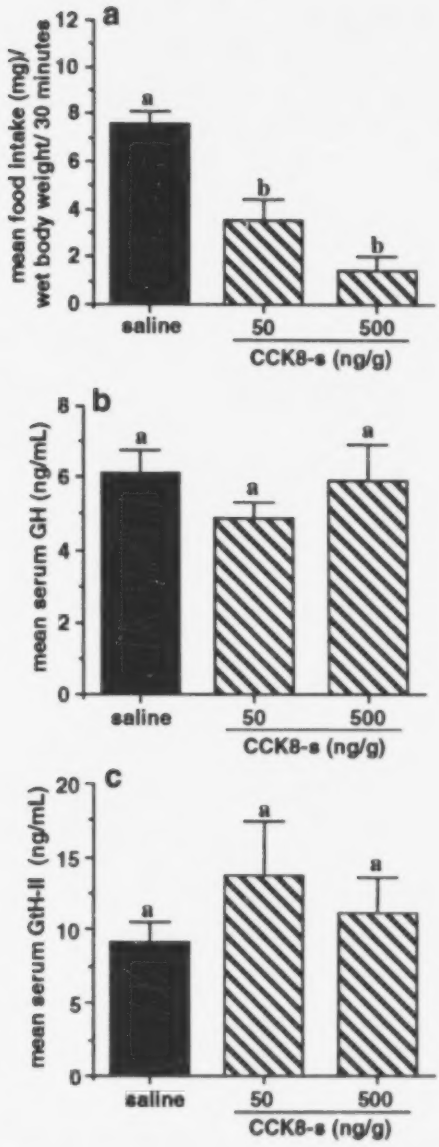

Fig. 5-4. Effects of intraperitoneal injection of CCK8-s on cumulative food intake (a), serum GH (b), and GtH-II (c) levels in goldfish. Fish were injected with either 50 or 500 ng/g CCK8-s (hatched bars), or saline (solid bar) and, at 15 minutes post-injection, fish were fed and cumulative food intake was recorded for 30 minutes. Blood samples were collected immediately following 30 minutes of feeding observations for hormone measurements (n = 8-9 fish/treatment).

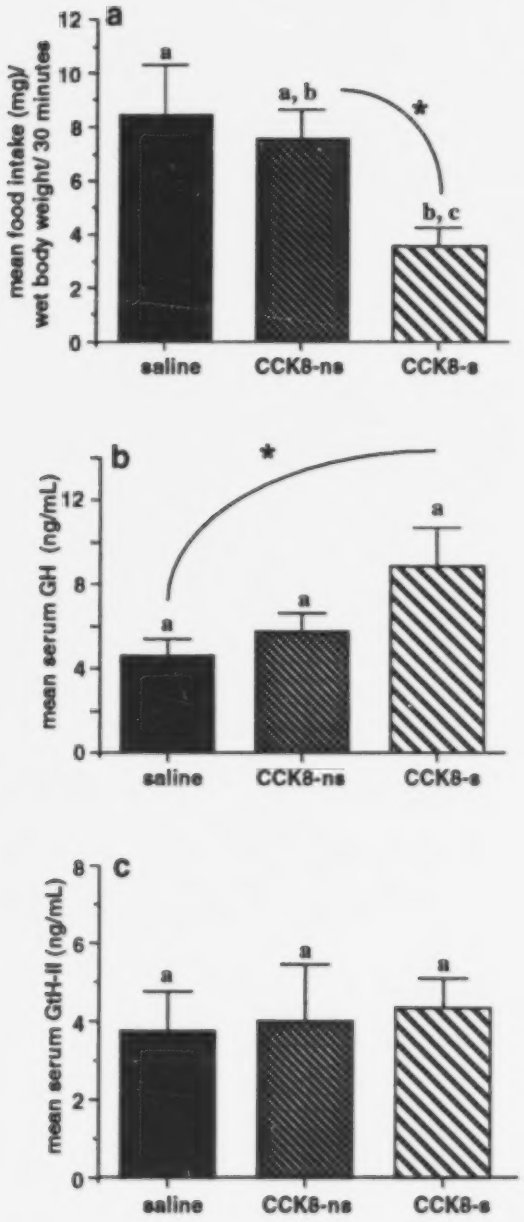

Fig. 5-5. Effects of intraperitoneal injection of CCK8-ns and CCK8-s on cumulative food intake (a), serum GH (b), and GtH-II (c) levels in goldfish. Fish were injected ip with equimolar doses of either CCK8-ns (95.6 ng/g) (narrow-hatched), CCK8-s (100 ng/g) (wide-hatched), or saline (solid bar) and, at 15 minutes post-injection, fish were fed and cumulative food intake was recorded for 30 minutes. Blood samples were collected immediately following 30 minutes of feeding observations for hormone measurements (n = 8-9 fish/group). Asterisk represents significance between groups with Student's t-test, $p < 0.05$.

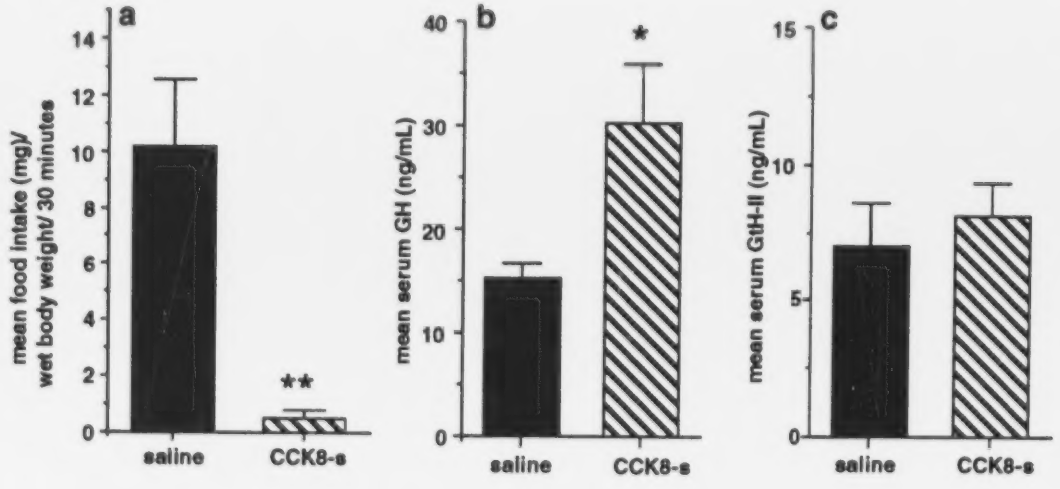

Fig. 5-6. Effects of intraventricular brain injection of CCK8-s on cumulative food intake (a), serum GH (b) and GtH-II (c) levels in goldfish. Fish were injected into the third brain ventricle with either CCK8-s (50 ng/g; hatched bar) or saline (solid bar) and, at 15 minutes post-injection, fish were fed and cumulative food intake was recorded for 30 minutes. Blood samples were collected immediately following 30 minutes of feeding observations for hormone measurements (n = 8 fish/treatment; Student's t-test, * $p < 0.05$; ** $p = 0.001$).

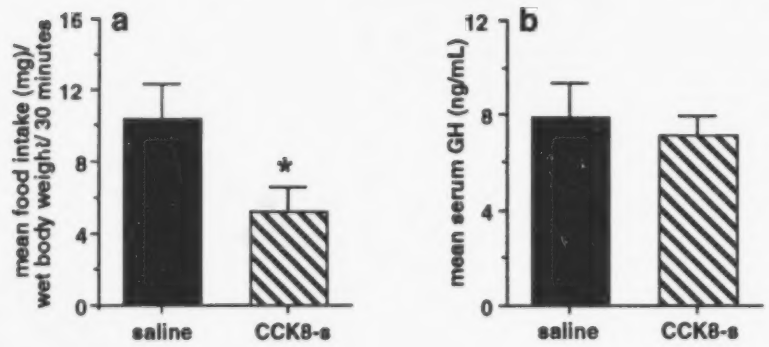

Fig. 5-7. Effects of intraventricular brain injection of CCK8-s on cumulative food intake (a), and serum GH (b) levels in goldfish. Fish were injected into the third brain ventricle with either CCK8-s (5 ng/g; hatched bar) or saline (solid bar) and, at 15 minutes post-injection, fish were fed and cumulative food intake was recorded for 30 minutes. Blood samples were collected immediately following 30 minutes of feeding observations for serum GH measurement (n = 8 fish/treatment).

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Chapter 6

CCK/Gastrin-Like Immunoreactivity in the Goldfish Pituitary and the Regulation of Pituitary Hormone Secretion by CCK-Like Peptides in vitro[1]

6.1 Introduction

In mammals, members of the CCK/gastrin family of peptides are known to be present in both endocrine cells of the gastrointestinal tract and within the central and peripheral nervous systems (Dockray, 1976; Vanderhaeghen *et al.*, 1980; Chapter 1). As may be expected from its widespread distribution, CCK/gastrin-like peptides have been implicated in the regulation of a diverse array of physiological events including gut motility, pancreatic secretion, gall bladder contraction, feeding behavior, and anterior pituitary hormone secretion (for reviews Morley, 1987; Beinfeld, 1988).

In lower vertebrates such as fish, CCK/gastrin-like peptides have also been reported in gastrointestinal tract and within the central nervous system. CCK/gastrin-like immunoreactive (IR) material has been described in gut endocrine cells of a number of fish species (Larsson and Rehfeld, 1978; Langer *et al.*, 1979; Holmgren *et al.*, 1982; Rombout and Taverne-Thiele, 1982; Jonsson *et al.*, 1987; Burkhardt-Holm and Holmgren, 1989), while IR nerve fibres have similarly been localized in the stomach, intestine and/or rectum of several fish, including the carp *Cyprinus carpio* (Bjenning and Holmgren, 1988), *P. reticulata* and *L. idus* (Langer *et al.*, 1979), and the sea scorpion, *Myoxocephalus scorpius* (Bjenning and Holmgren, 1988). CCK/gastrin-like IR has also been described in the brain and pituitary of several species, including the rainbow trout *Oncorhynchus mykiss* (Notenboom *et al.*, 1981), the green molly *Poecilia latipinna* (Batten *et al.*, 1986), the sea bass *Dicentrarchus labrax* (Moons *et al.*, 1992), and more recently the goldfish *Carassius auratus* (Himick and Peter, 1994; Chapter 5). Fibres staining for CCK/gastrin like IR have also been demonstrated within the proximal

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pars distalis (PD) of *O. mykiss* (Notenboom *et al.* 1981) and *P. latipinna* (Batten *et al.*, 1990). In *D. labrax*, Moons *et al.* (1988, 1992) described an abundance of CCK-like IR in nerve fibres within the proximal PD; these appeared to be associated with somatotropes.

Despite the growing immunohistochemical evidence which indicates that CCK/gastrin-like peptides are present within the central nervous system of teleosts, no studies have examined the possible role that CCK/gastrin-like peptides may play within the fish brain and pituitary. In Chapter 5 it was reported that CCK/gastrin-like peptides act centrally to regulate feeding behavior in goldfish. Since pituitary growth hormone (GH) and gonadotropin (GtH-II) were occasionally altered following peptide injection, it remains possible that CCK/gastrin-like peptides also act centrally to regulate the release of anterior pituitary hormone secretion. In mammals, studies indicate that CCK/gastrin-like peptides modify the release of GH and GtH *in vitro* (Morley *et al.*, 1979; Matsumura *et al.*, 1984).

The objectives of this chapter were to continue to investigate the actions of CCK/gastrin-like peptides in the central nervous system of the goldfish. Inititally, this involved examining the presence and distribution of CCK/gastrin-like IR within the goldfish pituitary. Subsequently, the effects of CCK/gastrin-like peptides on the release of GH and GtH-II from perifused goldfish pituitary fragments were examined. Data indicate that CCK-like peptides are co-distributed amongst the gonadotropes and somatotropes of the PPD and that CCK/gastrin-like peptides are capable of stimulating the release of both GtH-II and GH from perifused goldfish pituitary fragments.

6.2 Materials and Methods

Experimental Animals

Male and female goldfish (25-45 g) (Ozark Fisheries, Stoutland, MO) were maintained for approximately one month under simulated Edmonton natural photoperiod in 1.8 kL tanks which received a constant flow of aerated and dechlorinated city water at 17^{o}C. Fish were fed *ad libitum* twice daily with commercially prepared fish pellets until used for immunohistochemical studies or perifusion experiments. Fish were used in the seasonal phases of either gonadal regression or late recrudescence.

CCK/Gastrin-Like IR Peptides in the Goldfish Pituitary

Pituitary tissues of anesthetized goldfish were perfused *in situ* via the bulbous arteriosus with ice-cold 0.1 M phospate buffered saline (PBS; pH 7.4), followed by 4% paraformaldehyde containing 0.2% picric acid in PBS, pH 7.4. Pituitaries were removed, post-fixed and cryoprotected, and were then processed for cryostat sectioning following standard methods as outlined in Chapter 4.

To block non-specific binding of the CCK/gastrin antisera, pituitary sections were inititally incubated with 30% normal goat serum (Calbiochem, La Jolla, CA). This was followed by incubation with CCK/gastrin antisera G03 (1:300) for 18-20 hours at 4°C. Sections were subsequently exposed to goat anti-rabbit Immunoglobulin G, which was conjugated to tetramethylrhodamine isothiocyanate (TRITC; 1:100, Sigma, St. Louis, MO) for 60 minutes in the dark. Sections were mounted using carbonate-buffered glycerol/p-phenylenediamine (pH 8.5) and immunoreactive staining was photographed using a Zeiss fluorescence microscope. For use of avidin-biotin coupled with peroxidase as an immunoreactive label, endogenous peroxidase activity was intitally inactivated by incubating sections in methanol containing 0.3% hydrogen peroxide for 30 minutes. Following incubation in normal goat serum and exposure to CCK/gastrin antisera as outlined above, IR staining was examined using standard avidin-biotin immunoperoxidase procedures (ABC VectaStain Kit, Vector Labs, Burlingame, CA). Immunoreactivity was visualized as a brown reaction product following incubation with the chromagen diaminobenzidine (Sigma) in 0.05 M TRIS buffer (pH 7.6) containing 0.01% hydrogen peroxide. Immunoreactive staining was photographed using a Ziess light microscope. Alternatively, sections were also exposed to streptavidin (1:300; Calbiochem) for 30 minutes, followed by a 45 minute incubation of biotin-X conjugated to peroxidase (1: 500; Calbiochem). CCK/gastrin-IR was visualized as above.

Antisera and Specificity Controls

A description of the G03 rabbit anti-CCK/gastrin, kindly donated by Dr. A.C. Jonsson (University of Goteborg, Sweden), has been documented by radioimmunoassay (RIA) elsewhere (Jonsson *et al.*, 1987) and described in detail in Chapter 5. Staining by G03 was totally quenched when preabsorbed for 24 hours with CCK8-s (10 nmol/mL

diluted antisera) or gastrin 17-s (10 nmol/mL), but not when preabsorbed with bombesin (100 nmol/mL). Staining was abolished following the omission of primary antisera and replacement with normal rabbit serum, or following omission of secondary antisera. Rabbit anti-carp GtH-II, which is routinely used for GtH-II RIA measurement in our laboratory (Peter *et al.*, 1984; VanDerKraak *et al.*, 1992) was used at 1:20 000. Rabbit anti-carp GH, which is routinely used for GH RIA measurement in our laboratory (Marchant *et al.*, 1989) was used at 1: 35 000. Antisera to both of these pituitary hormones can be completely quenched following preabsorption with 10 nmol/mL of their respective antigen. To identify pituitary areas, serial sections were stained with cresyl violet.

Pituitary Perifusion System

The effects of CCK8-s on GtH-II and GH secretion from pituitary fragments of the goldfish were examined using a perifusion system described previously (Marchant *et al.*, 1987; Chapter 4). Fish were anesthetized, killed by spinal section, and the pituitaries were quickly removed and cut into fragments. Each perifusion column contained fragments equivalent to three pituitaries and fragments were perifused overnight with medium 199 containing Hank's Balanced Salts (Gibco, Grand Island, NY), followed by a 2 hour pre-perifusion of Hank's Balanced Salt Solution (HHBSA, supplemented with 25 mM HEPES and 0.1% BSA). In all experiments, the perifusion flow rate was 15 mL/h, and 5 minute (1.25 mL) fractions were collected using an automatic fraction collector. The samples were stored at -25^{o}C until hormone analyses. The concentration of GtH-II and GH levels in perifusion samples was determined by use of two specific RIAs developed for common carp GtH-II (Peter *et al.*, 1984; Van Der Kraak *et al.*, 1992) and common carp GH (Marchant *et al.*, 1989) (refer to Chapter 2 for details of RIA procedure).

Peptides Administered

Sulfated CCK8 (CCK8-s) was purchased from Richelieu Biotechnologies (Saint-Hyacinthe, QC), bombesin-14 (BBS) was purchased from Bachem Bioscience Inc. (Philadelphia, PA), and sulfated gastrin 17 (G17-s) was purchased fom Sigma Chem. Co. Synthetic salmon gonadotropin-releasing hormone (sGnRH) and nonsulfated CCK8

(CCK8-ns) were purchased from Pennisula Laboratories, Inc. sGnRH was diluted with HHBSA from stock solution (10 µg/20 µL in 0.1 N acetic acid) immediately prior to use in *in vitro* perifusion experiments.

Data Analyses and Statistics

For *in vitro* perifusion experiments, the GtH-II and GH release response to each pulse of CCK8-s was determined by quantifying the net response above average basal levels. Basal level was defined as the average hormone content in the three fractions collected immediately preceding each pulse (prepulse). Net responses were expressed as a percentage of the prepulse value. A release response was considered to be terminated when hormone content was within one standard error (SEM) of the mean prepulse value. The transformation to percentage of prepulse hormone levels allowed results from several columns to be combined for statistical analysis. Differences in the hormone responses to pulses of CCK8-s were compared by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. Differences between hormone responses in gonadal regressed fish and fish undergoing gonadal recrudescence were compared using Student's *t*-test. For all experiments, significance was considered to be at $p \leq 0.05$.

6.3 Results

6.3.1 CCK/Gastrin-Like IR in the Goldfish Pituitary

Using either immunofluorescence, ABC coupled to peroxidase, or streptavidin and biotin-X coupled to peroxidase, dense CCK/gastrin-like IR fibres were consistently observed within the proximal PD of the goldfish pituitary (Fig. 6-1 a and b). CCK/gastrin-like IR fibres within the proximal PD appeared to originate from the neurohypophysial stalk region (Fig. 6-2 a); bundles of CCK/gastrin-like IR fibres then branched into single fibres (Fig. 6-2 b). Here, immunoreactive staining was found to be co-distributed amongst both the IR-gonadotropes (Fig. 6-1 c) and IR-somatotropes (Fig. 6-1 d). CCK/gastrin-IR fibres appeared to overlay groups of somatotropes, but were also found to outline the periphery of groups of gonadotropes. Connections between neighbouring groups of cells created a "net" or "web"-like pattern of immunoreactive CCK/gastrin-like staining (Fig. 6-2 c and d). The extensive network of CCK/gastrin-IR

fibres amongst the gonadotropes and somatotropes of the goldfish proximal PD suggests that CCK/gastrin-like peptides probably communicate with both cell types.

CCK/gastrin-like IR fibres were also detected within the rostral PD, although the abundance of these fibres was substantially less than that observed within the PPD (Fig. 6-1a). In several instances, CCK/gastrin-like IR fibres were observed within the neurointermediate lobe (NIL) (not shown).

6.3.2 GtH-II and GH Release following Perifusion of Pituitary Fragments with CCK/Gastrin-Like Peptides

6.3.2.1 Effects of repeated administration of CCK8-s on GtH-II and GH release in recrudescent goldfish. Fig. 6-3 (a and b) represents the mean GtH-II and GH release responses from pituitary fragments of recrudescing goldfish which were exposed to repeated pulses of either 0.1 nM, 1.0 nM or 10 nM CCK8-s. Fig. 6-3 (c and d) summarizes the effects of repeated CCK8-s challenges on GtH-II and GH secretion when expressed as the mean total hormone release per pulse (expressed as a percent of basal prepulse levels). The initial challenge of 0.1, 1.0, or 10 nM CCK8-s resulted in a 42%, 226%, and 280% elevation in GtH-II secretion over basal, respectively. Independent of the dose, subsequent pulses of CCK8-s resulted in a similar magnitude of GtH-II release with respect to the initial pulse. The initial challenges of 0.1, 1.0, or 10 nM CCK8-s produced a 41%, 45%, and 162% increase in GH release, respectively. Repeated challenges of 0.1 and 1.0 nM CCK8-s stimulated GH release-responses of similar magnitudes; however, successive 10 nM CCK8-s challenges resulted in a significant decrease of the second, but not the third GH release-response. In all columns, a five minute control pulse of sGnRH at the end of the experiment stimulated both GtH-II and GH release.

6.3.2.2 Effects of repeated administration of CCK8-s on GtH-II and GH release in regressed goldfish. Fig. 6-4 (a and b) represents the mean GtH-II and GH release responses from pituitary fragments of regressed goldfish exposed to repeated pulses of 1.0 nM or 10 nM CCK8-s. Fig. 6-4 (c and d) summarizes the effects of repeated CCK8-s challenges on GtH-II and GH secretion when expressed as the mean hormone release per pulse. Initial challenges of either 1.0 or 10 nM CCK8-s resulted in 322% and 546%

increases in GtH-II secretion over basal, respectively. Regardless of the dose, subsequent pulses induced a GtH-II release response which was similar to the response obtained during the initial challenge. While a first pulse of 1.0 and 10 nM CCK8-s resulted in a 110% and 195% release of GH over basal, the third pulse of 10 nM CCK8-s produced a reduced release of GH to only 64% secretion above basal. In all columns, a five minute pulse of sGnRH stimulated GtH-II and GH release.

Based on the above experiments, an overall dose response effect of CCK8-s on pituitary GtH-II in gonadal recrudescing and regressed goldfish was determined. The GtH-II release responses (% over basal) to different doses of CCK8-s within and between perifusion columns were pooled, since there were no differences in the GtH-II responses to repeated challenges of CCK8-s within a column. Overall, pituitary fragments from regressed goldfish exhibited a greater release response of GtH-II to 1.0 nM or 10 nM CCK8-s relative to fragments from recrudescent fish (Fig. 6-5 a). In addition, a dose-response effect was observed following challenge of 1.0 nM and 10 nM CCK8-s on fragments from regressed fish (Fig. 6-5 a).

An overall dose response effect of CCK8-s on pituitary GH release was also determined following perifusion of fragments from fish of both sexual stages. Since some desensitization of the GH response may occur in fragments from fish of both sexual stages following successive challenges of 10 nM CCK8-s, only the GH release response obtained during the first peptide pulse was used. For the GH responses after perifusion of 1.0 nM CCK8-s, data were pooled from all three successive challenges, since there were no significant differences between the three release responses. While a dose-dependent release of GH was evident following perifusion of fragments of recrudescing fish with 1.0 nM and 10 nM CCK8-s, there were no significant differences between the GH responses to any one dose between recrudescing or regressed goldfish (Fig. 6-5 b).

An experiment was also conducted to examine the GtH-II and GH responses by pituitary fragments from gonadal regressed goldfish following five repeated pulses of 10 nM CCK8-s (data not shown). While each of the five repeated CCK8-s challenges resulted in a similar degree of GtH-II release (range 256% - 337%), GH desensitization again occurred following the initial pulse of CCK8-s.

6.3.2.3 Effects of increasing doses of CCK8-s on GtH-II and GH release in regressed goldfish. Fig. 6-6 (a and c) represents the mean GtH-II and GH responses of pituitary fragments from regressed goldfish to increasing doses of CCK8-s (0.1 nM to 100 nM). Fig. 6-6 (b and d) summarize the effects of increasing doses of CCK8-s on GtH-II and GH secretion respectively, when expressed as the mean total release of hormone per pulse. Increasing pulses of CCK8-s resulted in significantly greater GtH-II responses ranging from 28% (0.1 nM) to 516% (100 nM) above basal levels. While adminstration of CCK8-s pulses significantly increased the release of GH above basal levels, no significant differences existed in the responses between the doses tested. In all columns, a five minute pulse of 100 nM sGnRH stimulated GtH-II release.

6.3.2.4 Effects of CCK-like peptides on GtH-II and GH release. Fig. 6-7 (a and c) represents the mean GtH-II and GH responses of pituitary fragments from regressed goldfish to 5 minute pulses of 10 nM CCK8-s, BBS, CCK8-ns, G17-s, and sGnRH; CCK8-s, BBS, CCK8-ns and G17-s were administered in the order shown in Figure 7 (a and c), as well as in alternate orders with similar results. Fig. 6-7 (b and d) summarize the effects of CCK-like peptides on GtH-II and GH secretion respectively, when expressed as the mean total release of hormone per pulse. Both CCK8-s and G17-s were equal in their GtH-II releasing abilities, and produced overall greater responses relative to CCK8-ns. Similarly, both CCK8-s and G17-s showed greater capacity to stimulate GH release relative to CCK8-ns. With the exception of sGnRH, perifusion of BBS, a peptide unrelated to the CCK/gastrin family, caused the greatest GH response.

6.3.2.5 Dose response effects of CCK8-s on serum GH and GtH-II levels. Fig. 6-8 and Fig. 6-9 represent serum GH (a) and GtH-II (b) levels following ip injection of increasing doses of CCK8-s. In experiment 1 and 2 (Fig. 6-8 and Fig. 6-9, respectively), fish were randomly sorted into 4 tanks which received a constant flow of aerated and dechlorinated city water at 17°C. Fish were acclimated under Edmonton natural photoperiod and fed once daily a prescribed 2% body weight ration.

In experiment 1, groups of gonadal regressing fish were injected with either saline or one dose of CCK8-s (0.01, 0.1 or 1.0 μg/g). Fish were replaced back into their

respective tanks and allowed to recover fish. At 2.0 h post-injection, all 4 groups of fish were anesthetized and sampled for blood (n = 13 fish/group).

In experiment 2, groups of early recrudescing fish were injected with either saline or one dose of CCK8-s (0.001, 0.01, or 0.1 μg/g). Fish were replaced back into their respective tanks and allowed to recover. At 1.5 h post-injection, all 4 groups of fish were anesthetized, sampled for blood, and serum collected. In both experiments, serum GH and GtH-II levels remained unchanged.

6.4 Discussion

6.4.1 CCK/Gastrin-Like IR in the Goldfish Pituitary

This paper demonstrates that regardless of the technique employed (immunofluorescence or avidin-biotin coupled to peroxidase), use of antisera specific for CCK/gastrin-like peptides containing the COOH-terminal tetrapeptide sequence results in dense CCK/gastrin-like IR staining within the proximal PD. Immunoreactive fibres entered the goldfish anterior pituitary via the neurohypophysial stalk, and branching occurred to the point where individual fibres were evident. Within the proximal PD, CCK/gastrin-IR fibres were co-distributed amongst both the IR-gonadotropes and IR-somatotropes; connections between groups of these cells created a "net" or "web"-like pattern of immunoreactive CCK/gastrin-like staining. This unique intertwining of CCK/gastrin-like IR fibres amongst the gonadotropes and somatotropes likely provides an efficient means whereby hormone release and subsequent communication between the two cell types can be regulated by CCK/gastrin-like peptides within the fish anterior pituitary. Additionally, a small number of CCK/gastrin-like IR fibres were detected within the rostral PD, although staining was much weaker in intensity. No CCK/gastrin-like IR cell bodies were detected in any region of the pituitary.

CCK/gastrin-like IR material has been described in the anterior pituitary of several other fish species. In the rainbow trout, Notenboom *et al.* (1981) described the presence of intense CCK/gastrin-like IR material within fibre bundles of the proximal PD. In their study, CCK/gastrin-like IR pituitary fibres appeared to originate from the NLT of the caudal hypothalamus. Within the proximal PD, these fibre bundles branched and terminated on the basal lamina separating the neurohypophysis from the

adenohypophysis. In the sea bass *D. labrax*, Moons *et al.* (1988; 1992) have described CCK/gastrin-like IR nerve fibres within the proximal PD and in close proximity to somatotropes. The IR staining pattern described in these two fish species closely resembles our findings in the goldfish. In addition, unpublished studies in our laboratory confirm a similar pattern of CCK/gastrin-like IR staining within the NLT of the ventro-caudal hypothalamic region of the goldfish brain, further supporting Notenboom *et al.* (1981) that CCK/gastrin-like material in the fish pituitary originates from the ventral hypothalamus.

6.4.2 CCK/Gastrin-Like Peptides Release GtH-II and GH from the Goldfish Pituitary

Intense CCK/gastrin-like IR within the proximal PD amongst the gonadotropes and somatotropes suggests a role for CCK/gastrin-like peptides in the regulation of GtH-II and GH release. Results in the present study demonstrate that CCK8-s acts as a secretagogue in releasing both GtH-II and GH in the goldfish pituitary. When pituitary fragments were perifused with five minute pulses of CCK8-s at doses ranging from 0.1 nM to 100 nM, a dose-dependent release in GtH-II occurred in both gonadal recrudescing and regressed goldfish. Additionally, a dose-dependent effect of CCK8-s on GH release was evident in fragments from recrudescing goldfish. However, some desensitization of the GH release response occurred following administration of successive pulses of 10 nM CCK8-s to pituitary fragments from fish of both sexual stages. Using a similar protocol, desensitization of the GH response to neuropeptide Y (NPY) has also been documented in perifused goldfish pituitary fragments (Peng *et al.* 1990). While it is unclear how the desensitization to CCK8-s occurs, it is known that the releasable pool of stored GH is not depleted from fragments as evidenced by the response to sGnRH challenge at the end of the experiment. It remains possible that changes at the level of the receptor have occurred to produce the GH desensitization response to CCK8-s, and that in the case of recrudescent fish some recovery has occurred during the third challenge of CCK8-s.

The direct stimulation of GH and GtH-II release from goldfish pituitary fragments following perifusion of CCK-like peptides parallels findings in several studies on mammals. For example, Morley *et al.* (1979) demonstrated an acute release of both GH

and luteinizing hormone from cultured rat pituitary quarters following incubation with CCK8. Similarly, Matsumura *et al.* (1984) reported a dose-dependent stimulation of CCK8 on GH release from dispersed cells of the rat anterior pituitary. In lower vertebrates, a possible relationship between CCK and GtH has been proposed in the clawed toad *Xenopus laevis*, since gastrin/CCK-like activity was demonstrated in the infundibularis ventralis of the hypothalamus, an area proposed to be the origin of GtH-releasing hormone (Doerr-Schott *et al.*, 1981). Presently, the site of CCK action within the goldfish pituitary remains unknown; however, the extensive distribution of CCK/gastrin-like IR amongst both the gonadotropes and somatotropes strongly suggests a direct action at the level of both these cell types. Alternate to a direct action of CCK on gonadotropes and somatotropes, CCK may also interact with other hypothalamic regulators of GtH-II and GH release. The teleost pituitary is directly innervated by both aminergic and peptidergic fibres (Ball, 1981; Peter *et al.*, 1990), and these terminals are still present and functional within the pituitary fragment perifusion system used in the present study.

Pituitary fragments from regressed goldfish were more responsive than those from recrudescing fish in releasing GtH-II, but not GH, to either 1.0 nM or 10 nM CCK8-s. These findings are not unique since, in other studies using goldfish, seasonal effects of neuropeptides and neurotransmitters on GtH-II release have been reported. For example, Kah *et al.* (1992) described elevated serum GtH-II levels in only gonadal regressed or early recrudescing goldfish, but not in fish in late gonadal recrudescence, following intraperitoneal injection of γ-amino-butyric acid (GABA). When the direct actions of GABA on pituitary GtH-II release were examined *in vitro*, it was found that an interaction with estradiol existed. Additionally, Trudeau *et al.* (1993) showed that both testosterone and estradiol interact with sGnRH to modify the release of GtH-II from goldfish pituitary fragments *in vitro*. Peng *et al.* (1990) also reported a seasonal effect of NPY on the release of GtH-II following perifusion in goldfish pituitary fragments. Finally, the influence of gonadal sexual stage and seasonality on the GtH-II responses to sGnRH are well documented (for review Peter *et al.*, 1991). In the present study, pituitaries of regressed fish may be more responsive to CCK8-s relative to pituitaries of recrudescing fish, due to a lower basal steriod level present during the gonadal regressed

stage (Kobayashi *et al.*, 1986). In other higher vertebrates such as the rat, an interaction between CCK and estradiol has been documented, where estrous cycle variations influence both the concentration of CCK and the binding of sulfated ^{125}I-CCK within the ventromedial hypothalamus (Micevych *et al.*, 1988).

The actions of several CCK-like peptides on GtH-II and GH release from goldfish pituitary fragments were examined using a similar protocol as that conducted for successive CCK8-s pulses. When the effects of the CCK-like peptides on hormone release were compared, the sulfated forms of both CCK8 and G17 were more effective in stimulating the release of both pituitary hormones than the nonsulfated form of CCK8, indicating the importance of tyrosine sulfation for enhanced activation of the CCK/gastrin receptor within the goldfish pituitary. While the nonsulfated form of gastrin 17 was not examined in the present study, results with respect to the effects of the three peptides investigated are in accordance with earlier findings reported in fish. Vigna and Gorbman (1977) demonstrated that the sulfated forms of CCK8 and G17 were equipotent and displayed a 1000 fold enhanced activity over the same nonsulfated peptides in the contraction of coho salmon gallbladder longitudinal muscle strips. Similarly, in *G. morhua*, the sulfated forms of CCK/gastrin peptides were more effective than the nonsulfated forms in inhibiting gastric acid secretion (Holstein, 1982). More recently, Aldman and Holmgren (1987) reported that sulfated CCK8 and caerulein were more potent than CCK8-ns and G17-s in inducing excitatory contractility of isolated gallbladder strips of *O mykiss*, while Rajjo *et al.* (1988) documented similar findings in gallbladder muscle strips from the bluegill *L. macrochirus*. Jonsson *et al.* (1987) also described increased contraction of isolated stomach muscle strips following administration of the sulfated forms of CCK8 and G17. Data presented here indicate that CCK8-s and G17-s were similar in their GtH-II releasing abilities, while G17-s was only slightly more effective than CCK8-s in releasing GH. These findings support the concept of a single "primitive" CCK receptor within the brain and periphery of ectotherms that interacts at high affinity with only sulfated forms of CCK and gastrin-like peptides (Vigna *et al.*, 1986; Vigna, 1993). Unlike the two distinct endotherm CCK receptors (CCK-A and CCK-B) present in birds and mammals, this primitive receptor does not appear to have any strict requirement for the positioning of the sulfated tyrosine residue (i.e., CCK peptides: position seven from the carboxyl terminus; gastrins: position

six from the carboxyl terminus).

Finally, experiments presented here demonstrate that peripheral injection of CCK8-s at doses which are sufficient to cause changes in goldfish feeding behavior (Chapter 5) result in unaltered serum GH and GtH-II levels. These data suggest that CCK peptides likely mediate their stimulatory effects on pituitary hormone release via direct neural innervation from the brain and through acting as a neuropeptide, rather than as a circulating hormone released from the gut.

The teleost anterior pituitary is unique among vertebrates in that it lacks a functional median eminence and hypothalamo-hypophysial portal system. Neurosecretory regulation of anterior pituitary hormone release in fish occurs through direct neural innervation from the hypothalamus (Ball, 1981; Peter and Fryer, 1983; Peter *et al.*, 1990). With the presence of a functional median eminence and portal blood system within the pituitary of other vertebrates, it appears that a shift in the distribution of CCK/gastrin-like IR to include the posterior pituitary occurred. For example in the bullfrog *Rana catesbeiana*, a high concentration of CCK/gastrin-like IR is found predominantly within the NIL whereas little CCK/gastrin-like IR is detectable within the anterior lobe (Beinfeld *et al.*, 1983). In the neural lobes of the bovine and rat pituitary, CCK-like peptides of hypothalamic origin are well documented (Beinfeld *et al.*, 1981; Palkovits *et al.*, 1984; Kiss, 1985), as is the regulation of oxytocin and vasopressin secretion by CCK (Beinfeld *et al.*, 1980; Vanderhaeghen *et al.*, 1981). In the present study the extensive localization of CCK-like peptides within only the proximal PD, amongst both the gonadotropes and somatotropes, indicates that CCK-like peptides likely serve an important role in regulating GtH-II and GH release in the goldfish. The dose-dependent effects of CCK8-s on GtH-II and GH release in goldfish pituitary fragments, demonstrated in this study, provides direct evidence that CCK/gastrin-like peptides play a role in pituitary function.

Fig. 6-1. Distribution of CCK/gastrin-like IR in the goldfish pituitary. **(a)** CCK/gastrin-like IR fibres were localized within the proximal pars distalis (PPD) of the goldfish pituitary using ABC coupled to peroxidase. In some instances, CCK-like IR was detected in the rostral pars distalis (RPD; small arrows), 40X; **(b)** Adjacent section of pituitary showing abolished CCK/gastrin-like IR staining following preabsorption with CCK8-s (control), 40X; Serial sections of pituitary displaying IR gonadotropes **(c)** and IR somatotropes **(d)** within the PPD, 40X; NIL = neurointermediate lobe; scale bar = 300 μm.

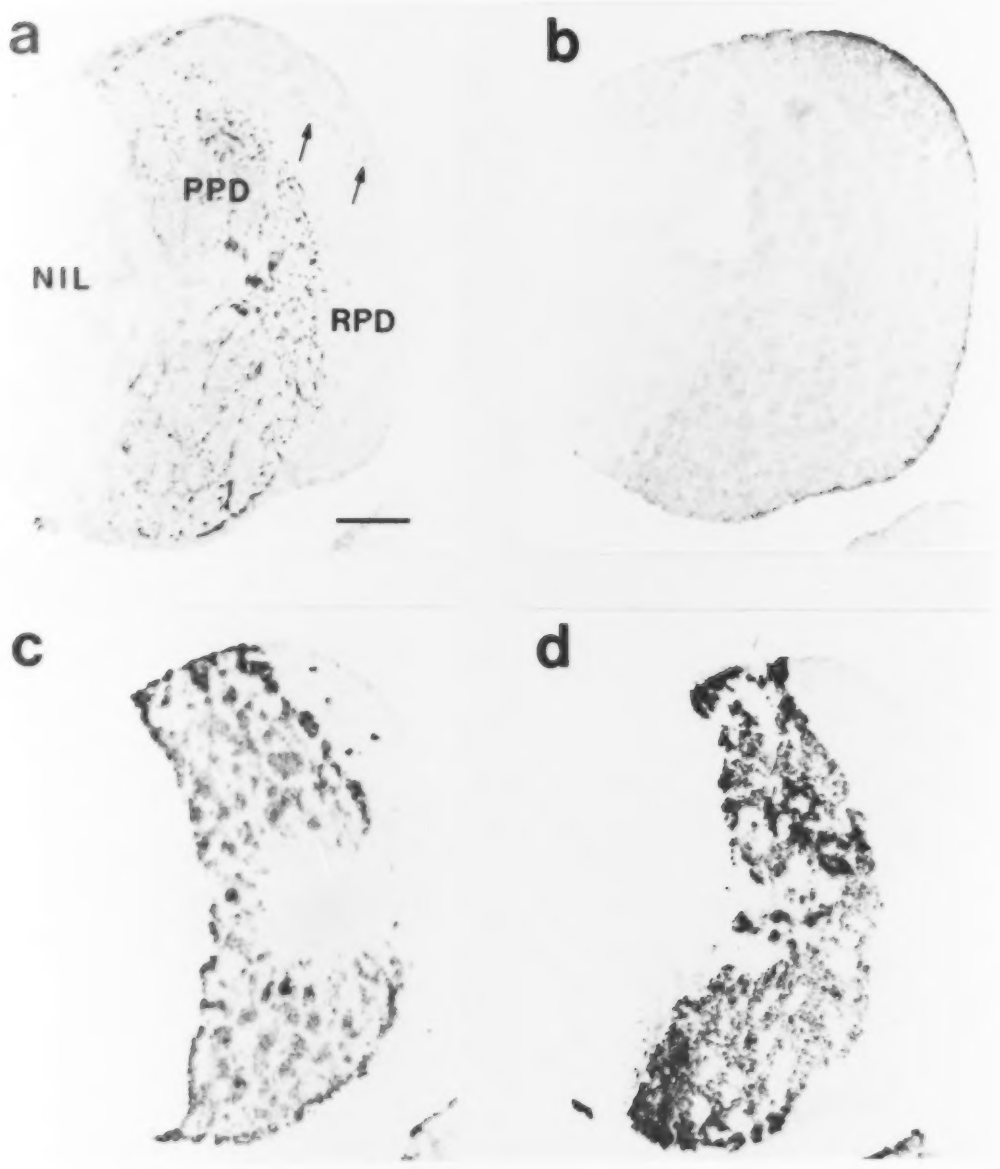
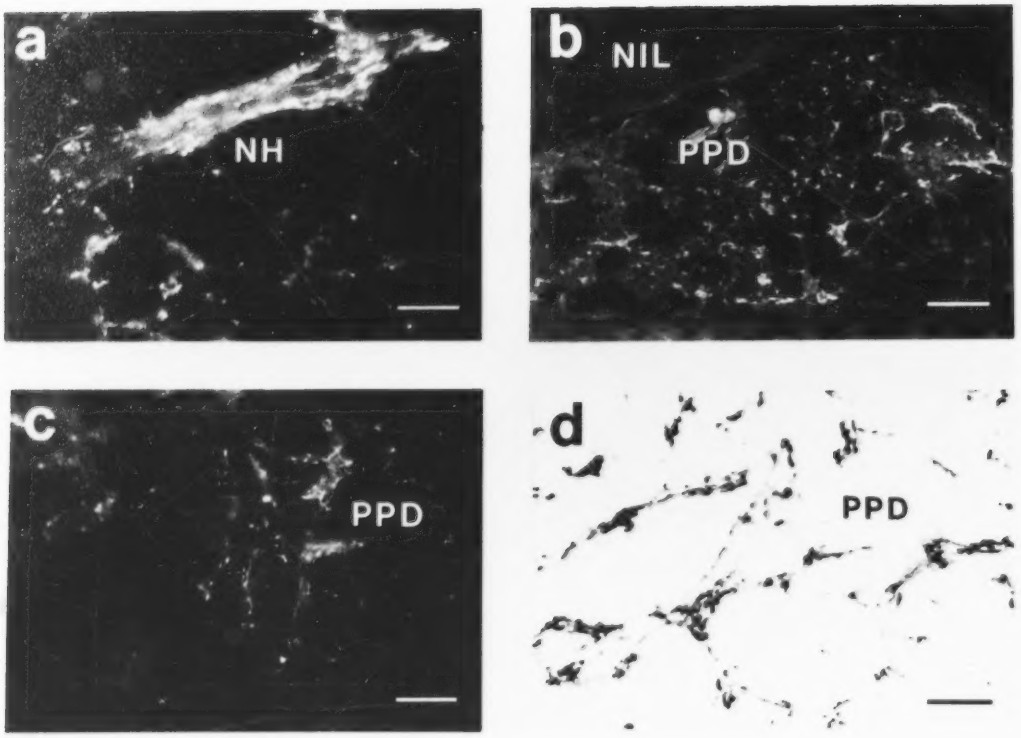

a
PPD
NIL
RPD
b
c
d

Fig. 6-2. CCK/gastrin-like IR in the proximal pars distalis of the goldfish pituitary. **(a)** Rhodamine-labeled CCK/gastrin-like IR fibre bundles present within the neurohypophysial (NH) stalk region. Fibres bundles originating from this region branched into individual fibres within the PPD, 400X, scale bar = 30 μm; **(b)** Rhodamine-labeled CCK/gastrin-like IR fibres within the PPD, 100X, scale bar = 80 μm; **(c)** Rhodamine-labeled CCK/gastrin-like IR fibres form links between groups of cells creating a "net"-like appearance of single fibres within the PPD, 400X, scale bar = 30 μm; **(d)** Distribution of CCK/gastrin-like IR fibres within the PPD following use of ABC coupled to peroxidase, 400X, scale bar = 30 μm.


a
NH
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PPD

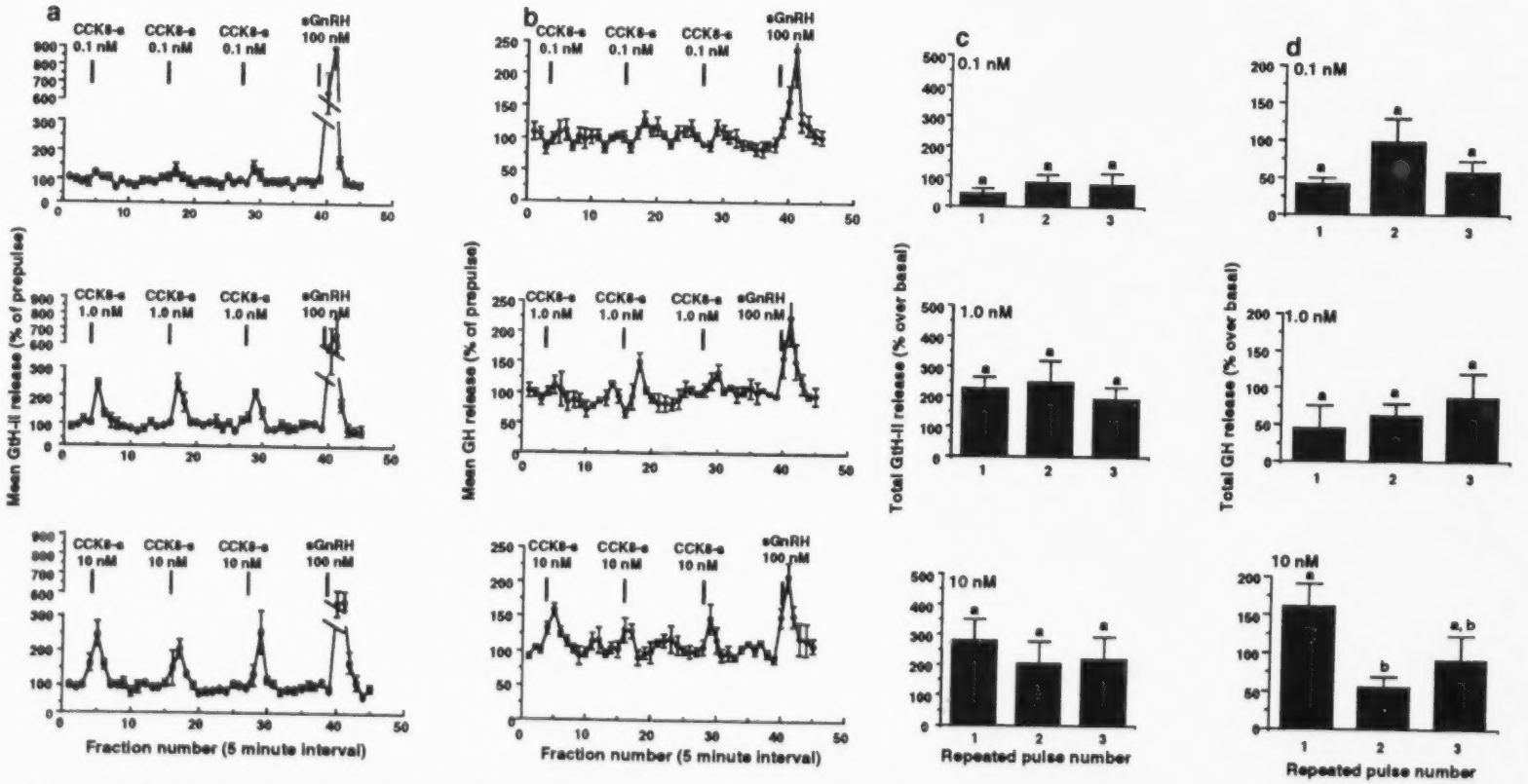

Fig. 6-3. Release profiles of GtH-II (a) and GH (b) in perifusion columns following repeated challenge with 0.1, 1.0 or 10 nM CCK8-s. Pituitary fragments from gonadal recrudescing goldfish were exposed to three 5 minute pulses of either 0.1 nM, 1.0 nM or 10 nM CCK8-s given at 55-minute intervals. At the end of the experiment, a 5 minute pulse of 100 nM sGnRH was administered as a control. Black bars represent application of peptide. The effects of CCK8-s on total GtH-II and GH release when expressed as the mean total hormone released per pulse are summarized in (c) and (d), respectively (n = 4 - 5 columns/dose; Duncan's multiple range test: lower case letters; mean hormone basal levels prior to peptide challenge, GtH-II 2.0 ng/mL; GH 14.4 ng/mL).

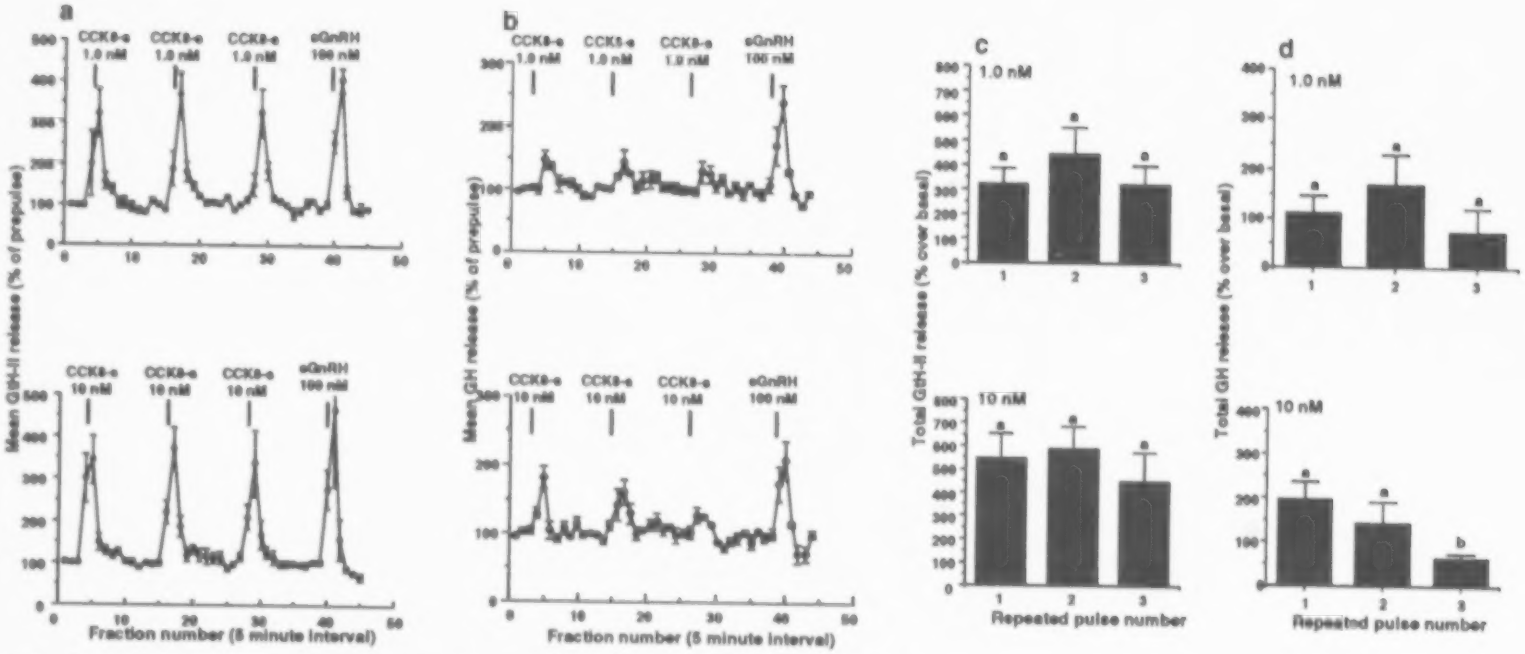

Fig. 6-4. Release profiles of GtH-II (a) and GH (b) in perifusion columns following repeated challenge with 1.0 or 10 nM CCK8-s. Pituitary fragments from gonadal regressed goldfish were exposed to three 5 minute pulses of either 1.0 nM or 10 nM CCK8-s given at 55-minute intervals. At the end of the experiment, a 5 minute pulse of 100 nM sGnRH was administered as a control. Black bars represent application of peptide. The effects of CCK8-s on total GtH-II and GH release when expressed as the mean total hormone released per pulse are summarized in (c) and (d), respectively (n = 5 columns/dose; Duncan's multiple range test: lower case letters; mean hormone basal levels prior to peptide challenge, GtH-II 1.3 ng/mL; GH 20.6 ng/mL).

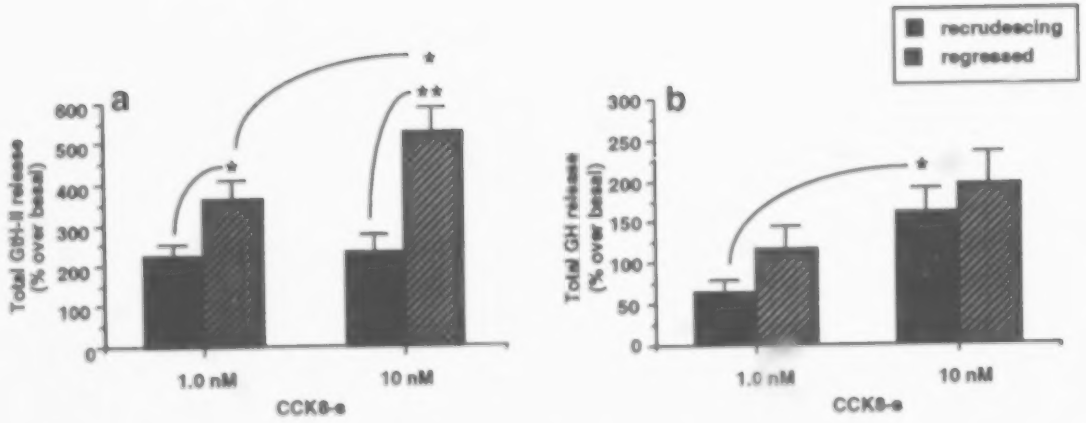

Fig. 6-5. The GtH-II (a) and GH (b) release responses of pituitary fragments from recrudescing or regressed goldfish following CCK8-s perifusion. Mean GtH-II release responses following CCK8-s challenges at 1.0 nM or 10 nM were pooled from two perifusion experiments to construct a dose response effect. Mean GH release responses, for pulse 1, only in each column following CCK8-s challenge at 1.0 nM or 10 nM were used to determine a dose response effect. Student's t-test: * = $p < 0.05$; ** = $p < 0.01$.

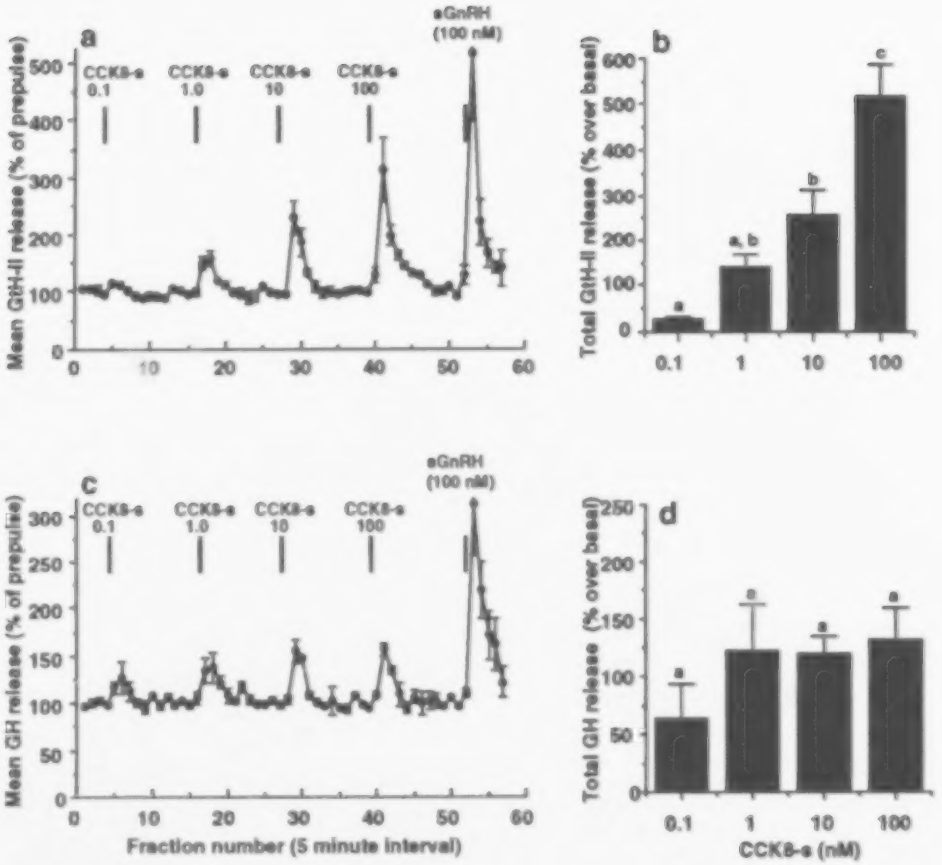

Fig. 6-6. Release profiles of GtH-II (a) and GH (c) in perifusion columns following challenge with increasing doses of CCK8-s. Pituitary fragments from gonadal regressed goldfish were exposed to four 5 minute pulses of increasing doses of CCK8-s (0.1 nM to 100 nM) given at 55-minute intervals. At the end of the experiment, a 5 minute pulse of 100 nM sGnRH was administered as a control. Black bars represent application of peptide. The effects of CCK8-s on total GtH-II and GH release when expressed as the mean total release of hormone per pulse are summarized in (b) and (d), respectively (n = 4 columns/dose; Duncan's multiple range test: lower case letters; mean hormone basal levels prior to peptide challenge, GtH-II 4.25 ng/mL; GH 23.3 ng/mL).

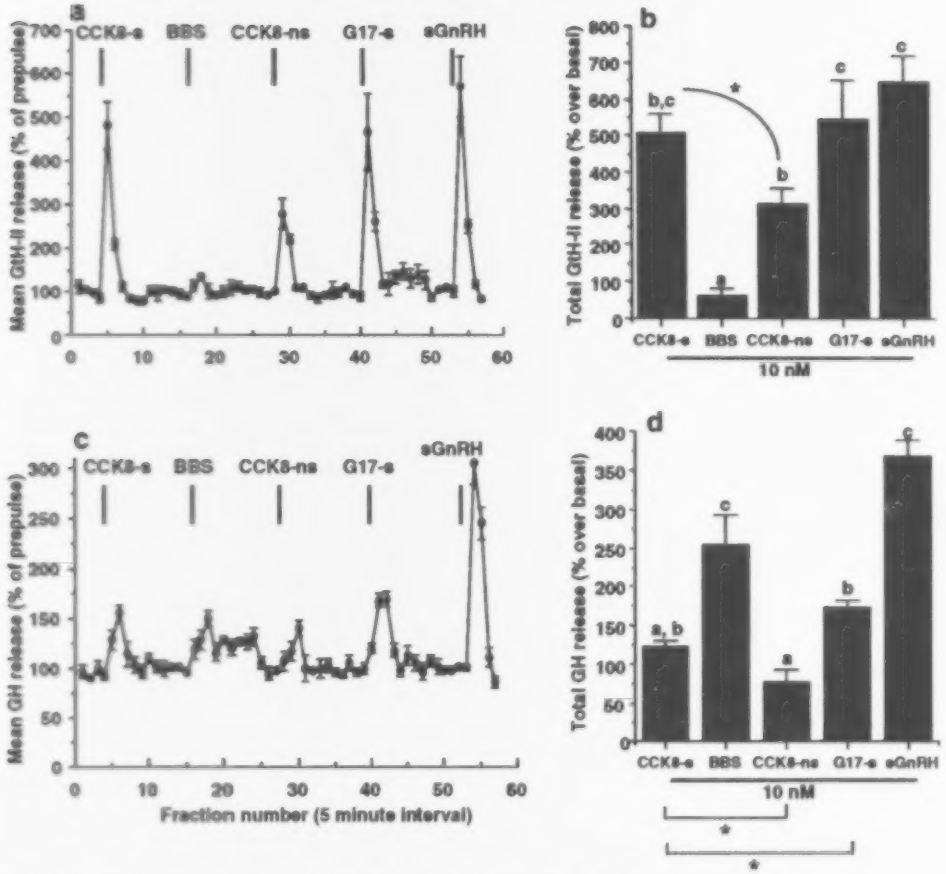

Fig. 6-7. Release profiles of GtH-II (a) and GH (c) in perifusion columns following challenge with CCK-like peptides. Pituitary fragments from regressed goldfish were exposed to 5 minute pulses of 10 nM CCK8-s, BBS, CCK8-ns, G17-s, and sGnRH given at 55-minute intervals. Black bars represent application of peptide. The effects of CCK8-s on total GtH-II and GH release when expressed as the mean total release of hormone per pulse are summarized in (b) and (d), respectively (n = 4 columns/dose; Duncan's multiple range test: lower case letters, Student's t-test: * = $p < 0.05$; mean hormone basal levels prior to peptide challenge, GtH-II 1.63 ng/mL; GH 4.03 ng/mL).

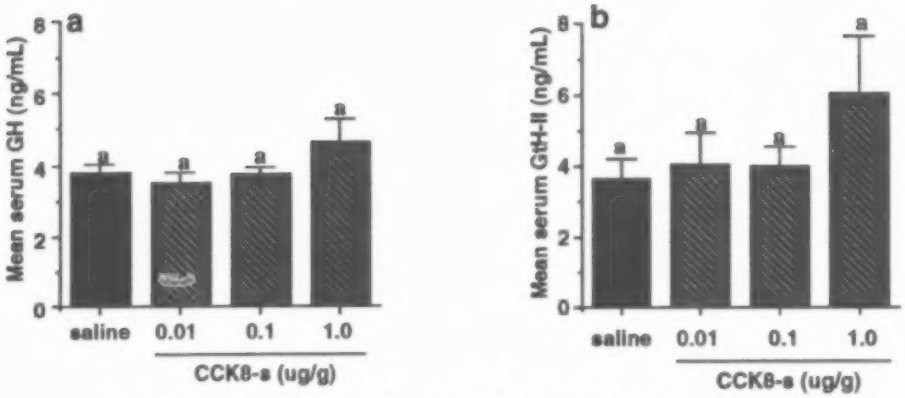

Fig. 6-8. Dose response effects of CCK8-s on serum GH (a) and GtH-II (b) levels (gonadal regressed). Groups of gonadal regressing fish were injected with either saline or one dose of CCK8-s (0.01, 0.1 or 1.0 ug/g). At 2.0 h post-injection, all 4 groups of fish were sampled for blood (n = 13 fish/group).

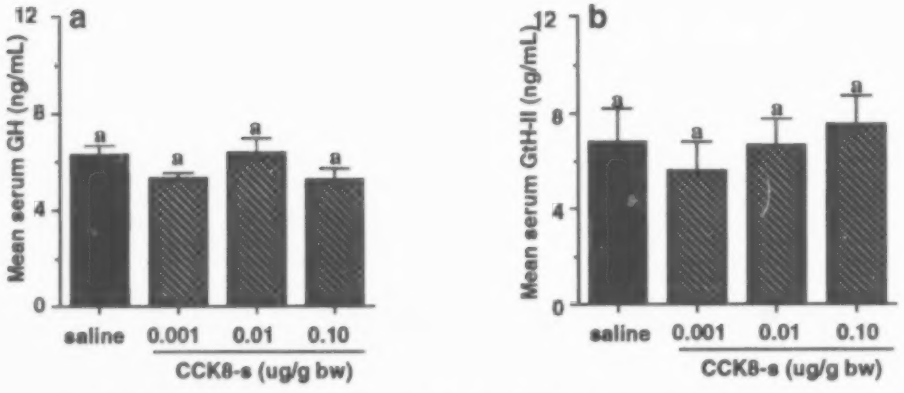

Fig. 6-9. Dose response effects of CCK8-s on serum GH (a) and GtH-II (b) levels (gonadal early recrudescent). Groups of gonadal early recrudescent goldfish were injected with either saline or one dose of CCK8-s (0.001, 0.01, 0.10 ug/g). At 1.5 h post-injection, all 4 groups of fish were sampled for blood (n = 11 fish/group).

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Chapter 7

Preliminary Studies on Bombesin Binding Sites in the Goldfish Central Nervous System

7.1 Introduction

Members of the bombesin (BBS) /gastrin releasing peptide (GRP) family of peptides are now recognized as regulators of feeding behavior and anterior pituitary hormone secretion within the central nervous system of the goldfish (Himick and Peter, 1994a; Himick and Peter, 1994b; Chapters 3 and 4). Additionally, studies in goldfish indicate the presence of BBS/GRP-like immunoreactive (IR) material throughout nerves and perikarya of the ventral forebrain, and within cells and nerve fibres of the anterior and posterior pituitary, respectively (Himick and Peter, 1994b; Chapter 4). Although these data provide strong support for a key role of BBS/GRP-like peptides within the teleost central nervous system, the sites of action whereby these peptides mediate their effects centrally remain unknown. Only one preliminary study has documented the presence of radiolabeled BBS binding sites in the antral stomach of a teleost, *Scorpaeichthys marmoratus* (Vigna and Thorndyke, 1989).

In mammals, putative BBS/GRP receptors have been characterized in both the peripheral and central nervous systems (Moody *et al.*, 1978; Westendorf and Schonbrunn, 1983; Houben and Denef, 1991; Vigna *et al.*, 1987), in pancreatic acinar cells (Jensen *et al.*, 1978; Swope and Schonbrunn, 1987), antral gastrin cells (Vigna *et al.*, 1989; Vigna *et al.*, 1990), human small cell lung carcinoma cell lines (Moody *et al.*, 1985), and Swiss 3T3 cells (Zachary and Rozengurt, 1985; Sinnett-Smith *et al.*, 1990). Furthermore, it is established that two classes of binding sites exist for BBS-related peptides (refer to Chapter 1). One receptor interacts selectively with BBS and GRP and the antagonist Ψ13, 14-BBS, but has low affinity for neuromedin B. Alternatively, the second binding site has a high affinity for neuromedin B, but lower affinity for Ψ13, 14-BBS, and very low affinity for BBS and GRP (Severi *et al.*, 1991). Of the two receptor types, the BBS/GRP binding site is more prevalent in distribution and appears to be widespread throughout the mammalian central nervous

system and gastrointestinal tract. The neuromedin B receptor is localized predominantly to the espophagus (Vigna *et al.*, 1987; Moran *et al.*, 1988), but may also be present in the brain and gut (Von Schrenck *et al.*, 1989; Lee *et al.*, 1990). Specific BBS/GRP receptors within the brain appear to be involved in the regulation of feeding behavior in mammals (Merali *et al.*, 1990; 1993; Laferrere *et al.*, 1992).

Preliminary studies in this chapter have examined the presence of BBS/GRP- binding sites in the goldfish brain and pituitary. The technique, *in vitro* receptor autoradiography, was employed in the present studies to localize ^{125}I-[Tyr-4]-BBS-14 binding sites within nuclei of goldfish brain areas, which cannot be accomplished through binding studies conducted in tissue homogenates.

7.2 Materials and Methods

Experimental Animals

Male and female goldfish (25-45 g) were purchased from Ozark Fisheries (Stoutland, MO) at several times of the year. Fish were maintained under simulated Edmonton natural photoperiod in 1.8 kL tanks which received a constant flow of aerated and dechlorinated city water at 17^{o}C. Fish were fed *ad libitum* twice daily with commercially prepared fish pellets until used for autoradiographic studies.

Tissue Preparation

Brain and pituitary tissues of anesthetized goldfish were quickly dissected and rinsed with ice-cold 0.1 M phospate buffered saline (PBS; pH 7.4). Tissues were immediately placed in Tissue-Tec embedding medium (Miles Inc, Elkhart, IN) and frozen on dry ice. Tissues were serially sectioned at 20 µm on a cryostat at -17^{o}C, thaw-mounted on microscope slides (Superfrost/Plus, Fisher Scientific), and stored in sealed slide boxes containing drierite ($CaSO_4$) at -20^{o}C until use.

Autoradiographic Technique

The autoradiographic technique used for localization of goldfish BBS/GRP binding sites has been described previously by Vigna *et al.* (1987). Briefly, slides containing tissue sections were allowed to thaw to room temperature in a sealed slide box containing drierite, and were then placed under vacuum for approximately thirty minutes. This latter

step enhanced tissue adherence to the slides, which was a problem due to the high lipid content of the unfixed goldfish brain tissues. The slide-mounted tissue sections were then preincubated in 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid (HEPES) pH 7.4, for 5 minutes at room temperature. Following this, sections were incubated in 10 mM HEPES, 130 mM NaCl, 4.7 mM KCl, 5 mM $MgCl_2$, 1 mM ethyleneglycol-*bis* (ß-aminoethylether)-N-N'-tetraacetic acid, 0.1% bovine serum albumin, 100 µg/mL bacitracin (pH 7.4), and 100 pM ^{125}I-[Tyr-4]-BBS-14 in the presence (control) or absence of 1 µM BBS for 60 minutes at room temperature. Conditions used in binding experiments were based on preliminary studies which revealed that incubation of sections with radiolabeled BBS for 60 minutes at room temperature resulted in excellent ^{125}I-[Tyr-4]-BBS-14 binding, with very little non-specific binding. The slide-mounted tissue sections were then washed four times for 5 minutes each in 10 mM HEPES with 0.1% bovine serum albumin (pH 7.4) at 4°C. Finally, the slides were rinsed twice for 5 seconds each at 4°C in distilled and deionized water. The slides were then dried at 4°C under a stream of cold air.

To visualize the localization of BBS receptors, the dried slides were placed in apposition to ß-Max High Performance Autoradiography Hyperfilm (Amersham, Oakville, ON) at room temperature for approximately 10 days. To eventually quantify the densities of radiolabeled binding sites in the brain and pituitary on the autoradiograms, a ^{125}I-microscale (20 µM; Amersham) was also exposed to the film. The film was then developed using Kodak D-19 Developer and fixed in undiluted Kodak Fixer. The film was washed under running tap water and hanged to dry. To identify brain nuclei, sections were stained with cresyl violet following exposure to autoradiography film.

Materials

^{125}I-[Tyr-4]-BBS-14 was obtained from Dupont, Wilmington, DE. BBS-14, BBS fragment (8-14), GRP 1-27, Neuromedin C (GRP 10), and Neuromedin B were purchased from Bachem California (Torrance, CA). Substance P and bacitracin were purchased from Sigma (St. Louis, MO).

Competitive Binding Studies

To determine the specificity of the BBS/GRP binding site in the goldfish central nervous system, preliminary competitive binding experiments were conducted to examine the ability of several BBS/GRP-related peptides to displace ^{125}I-[Tyr-4]-BBS-14 binding. Serial brain and pituitary sections were thaw-mounted on slides and then, following the above described autoradiographic technique, sections were incubated for 60 minutes at room temperature with a constant concentration of ^{125}I-[Tyr-4]-BBS-14 in the presence of one dose of 10^{-11} M to 10^{-6} M of the BBS/GRP-related peptides: BBS-14, GRP 1-27, neuromedin C (GRP 10), BBS fragment (8-14) and neuromedin B. Once the sections were washed, and subsequently dried under a stream of cold air, they were placed in apposition to ß-Max High Performance Autoradiography Hyperfilm.

Autoradiographic Analyses

In these preliminary experiments, only the presence and localization of ^{125}I-[Tyr-4]-BBS-14 binding sites in the goldfish central nervous system were examined. The use of ^{125}I-microscales, which were placed in apposition to the ß-Max High Performance Autoradiography Film at the same time as were the experimental slides, will allow future quantification of radiolabeled BBS binding in localized brain and pituitary regions through optical densitometric analyses. This will also allow me to eventually construct competitive binding curves, so that the binding affinities of various BBS/GRP-like peptides for the ^{125}I-[Tyr-4]-BBS-14 binding site can be calculated. To determine the specificity of the BBS/GRP binding site, visual analysis of the displacement abilities of several BBS/GRP-related peptides over the dose range of 10^{-11}M to 10^{-6}M were conducted. The dose at which partial and full displacement of ^{125}I-[Tyr-4]-BBS-14 binding occurred was determined, and from these data the affinity of the binding site for that particular peptide could be estimated.

To visually quantify the density of ^{125}I-[Tyr-4]-BBS-14 in localized goldfish brain and pituitary regions, a ranking system was used whereby greatest density (++++) to lowest density (+) of binding sites were estimated by visually inspecting autoradiographs of sections which had been incubated with radiolabeled BBS.

7.3 Results

Goldfish Pituitary

Autoradiography revealed binding sites for ^{125}I-[Tyr-4]-BBS-14 within the goldfish pituitary (Fig. 7-2a k; Table 7.1). ^{125}I-[Tyr-4]-BBS-14 binding was localized in the neurointermediate lobe (NIL), with lesser amounts of radiolabeled BBS binding also present in the anterior pituitary (Fig. 7-2a e, arrow). When serial sections of the pituitary were incubated in varying doses of BBS (10^{-6}M to 10^{-11}M) in the presence of ^{125}I-[Tyr-4]-BBS-14, full displacement of radiolabeled BBS was observed at 10^{-9}M BBS (Fig. 7-1a and b). Examination of the competitive binding of other BBS-related peptides revealed that GRP-1-27 and neuromedin C also caused full displacement of ^{125}I-[Tyr-4]-BBS-14 at 10^{-9}M. Only partial displacement of radiolabeled BBS occurred with BBS fragment (8-14) and neuromedin B at 10^{-7}M, while full displacement was obtained following incubation of BBS fragment (8-14) and neuromedin B at 10^{-6}M.

Non-specific binding of ^{125}I-[Tyr-4]-BBS-14 was negliglible, as determined by the addition of 10^{-6}M BBS (Fig. 7-2a l). Furthermore, no displacment occurred when sections were incubated with ^{125}I-[Tyr-4]-BBS-14 in the presence of 10^{-6}M Substance P. These preliminary studies provide evidence that the BBS/GRP-binding site in the goldfish pituitary is saturable and of high affinity for BBS/GRP-like peptides.

Goldfish Brain

Similar to findings in the pituitary, when serial sections of brain regions were incubated with varying doses of BBS (10^{-6}M to 10^{-11}M) in the presence of ^{125}I-[Tyr-4]-BBS-14, full displacement of radiolabeled BBS was observed in nearly all brain regions at 10^{-9}M BBS. Examination of the competitive binding studies conducted with several BBS-like peptides revealed that incubation of sections with GRP-1-27 and neuromedin C caused full displacement of ^{125}I-[Tyr-4]-BBS-14 at 10^{-9}M. Partial binding displacement occurred with BBS fragment (8-14) and neuromedin B at 10^{-7}M, while full displacement was obtained following incubation of 10^{-6}M BBS fragment (8-14) and neuromedin B with BBS radiolabel. Interestingly, a small number of brain regions contained ^{125}I-[Tyr-4]-BBS-14 binding which was not displaceable in the presence of BBS at 10^{-6}M to 10^{-11}M (Fig. 7-1b, arrows). In this case, it is possible

Table 7-1. ^{125}I-[Tyr-4]-BBS-14 specific binding sites and their relative densities in the goldfish CNS (note: only brain areas which could be identified are listed).

Brain Area		^{125}I-[Tyr-4] -BBS-14 Binding Density
Diencephalon:		
Olfactory Areas and Telencephalon		
OB	olfactory bulb (olfactory nerve)	++++
Vd	area ventralis telencephali pars dorsalis	+++
Vl	area ventralis telencephali pars lateralis	+
Vv	area ventralis telencephali pars ventralis	+++
Dc	area dorsalis telencephali pars centralis	+/++
Dd	area dorsalis telencephali pars dorsalis	+++
Dl	area dorsalis telencephali pars lateralis	+++
Dm	area dorsalis telencephali pars medialis	+++
Hypothalamus		
NAH	nucleus anterioris hypothalami	+
NAT	nucleus anterior tuberis	++/+++
NDLI	nucleus diffusus lobi inferioris	++ (a)/++++ (p)
NDTL	nucleus diffusus tori lateralis	++
NE	nucleus entopeduncularis	+++
NG	nucleus glomerulosus	+++
NLT	nucleus lateral tuberis	+++
NPO	nucleus preopticus	+++
NPT	nucleus posterior tuberis	+++
NPP	nucleus preopticus periventricularis	+++
NRL	nucleus recessus lateralis	+++
NRP	nucleus recessus posterioris	+++
Dorsal Diencephalon		
NH	nucleus habenularis	++++
NR	nucleus rotundus	++
NDL	nucleus dorsolateralis thalami	+++
NDM	nucleus dorsomedialis thalami	+++
NVM	nucleus ventromedialis thalami	+++
Midbrain and Hindbrain		
OTec	optic tectum	++++ (a)/+++ (p)
C	cerebellum (cortex)	+++ (a)/++++ (p)
NIp	nucleus interpeduncularis	++
TS	torus semicircularis	+
VL	vagal lobe	++
FL	facial lobe	+/++
Pituitary		
NIL	neurointermediate lobe	++++
PD	pars distalis	+

+ = lowest binding/++++ = highest binding observed; (a) = anterior, (p) = posterior

that ^{125}I-[Tyr-4]-BBS-14 binds to a second site in the fish central nervous system which is not a GRP/BBS peptide preferring site.

Specific binding of ^{125}I-[Tyr-4]-BBS-14 was localized throughout the goldfish brain. Radiolabeled BBS binding was detected in caudal regions of the olfactory bulbs (OB) and within the olfactory nerve (Fig. 7-2a a). ^{125}I-[Tyr-4]-BBS-14 binding was also detected in the area dorsalis telencephali pars medialis (Dm), the area dorsalis telencephali pars dorsalis (Dd), and the area dorsalis telencephali pars lateralis (Dl) (Fig. 7-2a b and c). Radiolabeled BBS binding sites were also present medially within the area ventralis telencephali pars ventralis (Vv) and the area ventralis telencephali pars lateralis (Vl) of the telencephalon (Fig. 7-2a b and c).

Specific binding of ^{125}I-[Tyr-4]-BBS-14 was also detected within the preoptic hypothalmus, including the nucleus entopeduncularis, nucleus preopticus periventricularis (NPP), and nucleus preopticus (NPO). In the ventro-posterior hypothalamus, specific radiolabeled BBS binding was localized to the nucleus recessus lateralis (NRL), nucleus glomerulosus (NG), and the nucleus lateral tuberis (NLT) (Fig. 7-2a e, f, g). Finally, ^{125}I-[Tyr-4]-BBS-14 binding was detected in the nucleus diffusis lobi inferioris (NDLI); visual inspection of autoradiographs indicated that density of these BBS radiolabeled binding sites increased from the anterior NDLI to the posterior NDLI (Fig. 7-2a g, h).

^{125}I-[Tyr-4]-BBS-14 specific binding was also detected in dorsal regions of the brain, including the optic tectum (OTec) (Fig. 7-2a d, f, g, h), the nucleus habenularis (NH), (Fig. 7-2a i, arrow), several thalamic nuclei, and within the nucleus rotundus (NR) (Fig. 7-2a d; NR = arrow). Within the central midbrain, BBS binding was also localized in the nucleus interpeduncularis (NIp) (Fig. 7-2a h, arrow), the torus semicircularis (TS), and the cortex of the cerebellum (Fig. 7-2a g and i). In the medulla of the hindbrain, ^{125}I-[Tyr-4]-BBS-14 binding sites were localized in the vagal lobe (VL) and the facial lobe (FL).

Non-specific binding was negliglible, as determined by the addition of unlabeled BBS at 10^{-6}M (Fig. 7-1a). These preliminary studies provide evidence that the BBS/GRP-binding site in the goldfish brain is saturable and of high affinity for BBS/GRP-like peptides.

7.4 Discussion

Data in this chapter represent preliminary studies to document the presence of ^{125}I-[Tyr-4]-BBS-14 specific binding sites within the central nervous system of the goldfish. These studies are the first to report that specific binding sites for BBS/GRP-like peptides exist within teleost fish, and to present findings which describe their general distribution within the goldfish brain and pituitary.

Based on binding displacement studies in brain and pituitary serial sections which were incubated with ^{125}I-[Tyr-4]-BBS-14 and varying doses of BBS at 10^{-6}M to 10^{-11} M, radiolabeled bound BBS was found to be fully displaced in the presence of 10^{-9}M BBS. Partial displacement of ^{125}I-[Tyr-4]-BBS-14 occurred when sections were incubated at 10^{-10}M BBS. Radiolabeled BBS binding was similarly found to be fully displaced by the BBS/GRP-like peptides GRP 1-27 and neuromedin C at 10^{-9}M. These findings demonstrate that the ^{125}I-[Tyr-4]-BBS-14 binding site in the goldfish central nervous system is specific for BBS and GRP peptides. Full displacement of ^{125}I-[Tyr-4]-BBS-14 by the BBS/GRP-like peptides neuromedin B and BBS fragment (8-14) required high concentrations of 10^{-7}M and 10^{-6}M, indicating that the BBS/GRP binding site within the goldfish brain and pituitary has a higher affinity for BBS/GRP-like peptides which contain the BBS/GRP carboxyl terminal sequence of greater than seven amino acid residues. Variations in this C-terminal sequence result in an approximate 10 to 100 -fold decrease in the binding affinity. Such findings suggest that the BBS/GRP binding site in fish is similar to the BBS/GRP-preferring receptor present in mammals; which has high affinity for BBS-like and GRP-like peptides, and lower affinity for neuromedin B (Severi *et al.*, 1991)

Interestingly, in a small number of brain regions, ^{125}I-[Tyr-4]-BBS-14 binding could not be displaced with 10^{-6} M BBS (Fig. 7-1 b). This suggests that a second BBS-related peptide binding site may be present in the goldfish central nervous system, which is unlike the widespread BBS/GRP-preferring binding site. This site may actually represent a neuromedin B preferring binding site, which has been documented in low densities within certain brain areas of mammals (Von Schrenck *et al.*, 1980; Lee *et al.*, 1990).

Based on the displacement studies, ^{125}I-[Tyr-4]-BBS-14 binding sites which were specific and of high affinity for BBS/GRP-like peptides were localized throughout the

goldfish forebrain, midbrain and hindbrain. In particular, areas within the OB and OB nerves, the medial and dorso-lateral telencephalon, the ventro-posterior hypothalamus, the OTec, and the cortex of the cerebellum, all contained high amounts of ^{125}I-[Tyr-4]-BBS-14 specific binding (refer to Table 7.1 for binding in localized nuclear areas). In addition, a high density of specific BBS binding was observed in the NIL of the goldfish pituitary, with detectable binding in the anterior pituitary.

In most cases, localization of specific ^{125}I-[Tyr-4]-BBS-14 binding sites in the goldfish brain and pituitary matched the distribution of BBS/GRP-like IR peptides described in the goldfish central nervous system (Himick and Peter, 1994b; Chapter 4). Several mismatches between the distribution of ^{125}I-[Tyr-4]-BBS-14 binding sites and the localization of BBS/GRP-like IR peptides were present, however. Most notable, was the observation that no BBS/GRP-like IR material was detected in the cerebellum, although ^{125}I-[Tyr-4]-BBS-14 binding sites were localized within the cortex of the cerebellum. Early anatomical and histological studies have demonstrated that the teleost cerebellum receives sensory information from both the posterior and anterior lateral-line nerves, and from highly developed optic tracts (Ariens Kappers *et al.*, 1965). In particular, the nucleus lateralis of the optic tract has been regarded as occasionally carrying gustatory impulses to the cerebellum (Ariens Kappers *et al.*, 1965). Such sensory input implicates the cerebellum as an integration center for receiving external stimuli. It is possible that BBS within the cerebral spinal fluid has access to these cerebellar ^{125}I-[Tyr-4]-BBS-14 binding sites and through this route, BBS acts to modulate incoming feeding-related sensory signals, subsequently altering feeding behavior. The role of BBS/GRP-like peptides in the integration within the brain of external stimuli is also supported by the findings of ^{125}I-[Tyr-4]-BBS-14 binding sites in both the OTec, and the OB and olfactory nerve, which receive visual and olfactory stimuli from the environment, respectively.

Based on earlier anatomical studies illustrating gustatory and medial forebrain bundle connections to the inferior lobe of the hypothalamus (Herrick, 1905; Crosby and Showers, 1969), as well as electrical stimulation studies on specific brain areas in the carp *Cyprinus carpio* (Redgate, 1974), bluegill sunfish *Lepomis macrochirus*, and in tilapia *Tilapia macrocephala* (Demski and Knigge, 1971; Demski, 1973; for review

Demski, 1981; 1983), areas within the fish ventro-posterior hypothalamus and the hypothalamic inferior lobes appear to be involved in the regulation of feeding activity (after Demski 1983, for review Chapter 1). In the present study, ^{125}I-[Tyr-4]-BBS-14 binding sites were detected in this hypothalamic feeding area. Such findings, as well as the presence of BBS/GRP-like IR material in the ventro-posterior hypothalamus and the hypothalamic inferior lobes (Himick and Peter, 1994), and the suppressive actions of BBS on feeding behavior following intraventricular brain injection into goldfish (Himick and Peter, 1993), all provide strong support for a role of BBS/GRP-peptides in the central regulation of feeding behavior in the goldfish. It is postulated that during food intake in fish, BBS/GRP-like peptides are released locally in these brain regions; these peptides then bind to their specific, high affinity binding sites located within nuclei of the ventro-posterior hypothalamus and inferior hypothalamic lobes. Concomitantly, BBS may also be released peripherally from within the gut during feeding, and subsequently bind to the specific gut mucosal BBS/GRP binding sites which have also been reported in the present chapter (Fig. 7-2a j).

^{125}I-[Tyr-4]-BBS-14 specific binding sites were also detected in the VL and FL of the goldfish hindbrain. In cyprinids, the VL is involved in the discriminative selection of ingested food, through VL sensory input via vagal innervation of the oropharygeal cavity and palatine organ (Kanwal and Finger, 1992). Likewise, the FL performs discriminative sensorimotor functions leading to the selective ingestion of food, through FL sensory input via the facial nerve innervation of the outer skin surface and lips (Kanwal and Finger, 1992). Together these brain areas likely act to receive sensory information about the feeding environment and transmit signals to the hypothalamic feeding center, where food intake is altered accordingly. In the goldfish, these two brain areas also contain BBS/GRP-like IR peptides (Himick and Peter, 1994b; Chapter 4), and it is hypothesized that endogenous BBS is released within the hindbrain following gustatory input signals to the medulla; BBS then binds to its high affinity receptors located in this region and acts to alter feeding activity. Areas of the hindbrain containing ^{125}I-[Tyr-4]-BBS-14 binding are also exposed to the peripheral circulation, and it is possible that peripherally BBS- mediated feeding suppression in the goldfish (Himick and Peter, 1994a; Chapter 3) may be mediated through the binding of circulating BBS to hindbrain receptors.

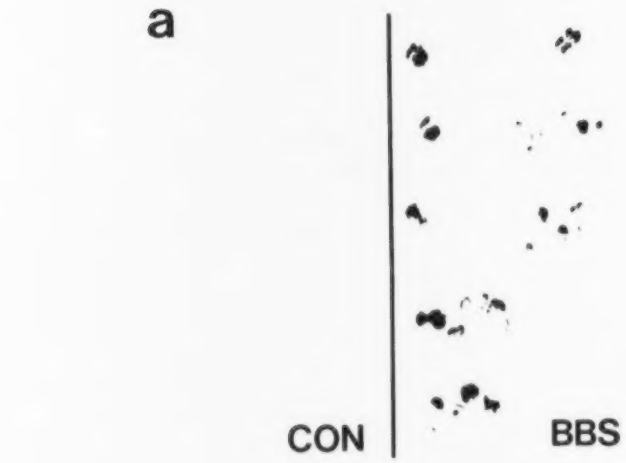
Finally, preliminary studies presented in this chapter demonstrate the presence of ^{125}I-[Tyr-4]-BBS-14 specific binding sites in the NIL of the goldfish pituitary. It appears that in the goldfish, BBS/GRP-like peptides play a fundamental physiological role within the NIL, based on findings of both a high concentration of BBS/GRP-like IR material in the NIL (Chapter 4) and the presence of specific, high affinity binding sites reported in this pituitary region (present chapter). Anatomically, the NIL represents an interdigitation between the pars intermedia and the pars nervosa; melanocyte stimulating hormone (MSH) is present in the pars intermedia, while the nonapeptides arginine vasotocin and isotocin are produced within the nucleus preopticus of the hypothalamus, and are released from the pars nervosa region of the NIL (Peter and Fryer, 1983). The presence of both BBS/GRP-like IR and ^{125}I-[Tyr-4]-BBS-14 binding sites in the NIL indicate that BBS/GRP-like peptides may play a role in regulating MSH, or arginine vasotocin and isotocin secretion from the fish pituitary.

Low amounts of specific ^{125}I-[Tyr-4]-BBS-14 binding were also present in the goldfish anterior pituitary. Such findings would explain results obtained in Chapter 4, which document the release of low amounts of both growth hormone and gonadotropin from the proximal pars distalis following perifusion of goldfish pituitary fragments with BBS-related peptides (Himick and Peter, 1994b).

Overall, preliminary studies presented in this chapter demonstrate that ^{125}I-[Tyr-4]-BBS-14 binding sites are present within the goldfish central nervous system. Based on competitive binding studies, these BBS binding sites are specific and of high affinity for BBS/GRP-like peptides. The localization of specific BBS binding sites within areas of the ventro-posterior hypothalamus and hypothalamic inferior lobes provides further evidence for a central role of BBS/GRP-like peptides in the regulation of feeding behavior in goldfish. The widespread distribution of ^{125}I-[Tyr-4]-BBS-14 binding sites in other brain areas associated with sensory input, such as the OB and olfactory nerve, the telencephalon, the OTec, and the VL and FL of the hindbrain, indicate that BBS/GRP-like peptides likely act in brain areas which receive sensory information about the environment and external feeding cues. BBS/GRP-like peptides may act as integrative peptides in the brain by receiving such sensory stimuli and transmitting information to the hypothalamic feeding area. The presence of specific and high affinity

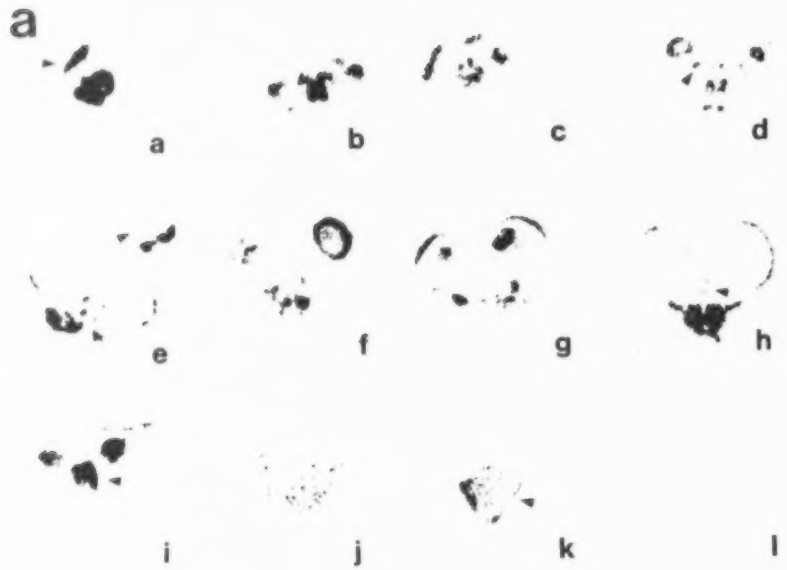
BBS binding sites in the goldfish pituitary also provides additional evidence for a role of BBS/GRP-like peptides in the regulation of pituitary hormone secretion.

Fig. 7-1. Binding sites for ^{125}I-[Tyr-4]-BBS-14 in the goldfish central nervous system. **a)** Goldfish brain sections exposed to ^{125}I-[Tyr-4]-BBS-14 in the presence (CON) or absence (BBS) of 10^{-6}M BBS. ^{125}I-[Tyr-4]-BBS-14 binding in brain and pituitary sections was displaced following incubation of radiolabeled BBS in the presence of 10^{-6}M BBS. **b)** Typical competitive binding study of ^{125}I-[Tyr-4]-BBS-14 in goldfish brain and pituitary sections, which demonstrates displacement of BBS radiolabel in the presence of BBS at 10^{-9} M to 10^{-7}M (upper half of slide). Note that few brain areas contain binding sites for ^{125}I-[Tyr-4]-BBS-14 which are not displaceable with BBS ligand (arrows on lower half of slide).


a
CON
BBS

b
-7
-8
-9
-10
-11

Fig. 7-2. Distribution of ^{125}I-[Tyr-4]-BBS-14 binding sites in the goldfish brain, pituitary and gut. **a)** Representative sections of goldfish brain, pituitary and gut which demonstrate ^{125}I-[Tyr-4]-BBS-14 binding sites in: a) OB and olfactory nerve (arrow), b and c) Vv, Vd, Dl and Dd areas of telencephalon, d) NLT, and OTec, NR (arrow), and thalamic areas, including NVM, of dorsal diencephalon, e) NDLI and anterior (arrow) and NIL regions of posterior pituitary, f) OTec and NLT regions, g) OTec, cortex of cerebellum, and nuclei within the ventro-posterior hypothalamus, including the NRL and NG regions, h) caudal level of NDLI, NIp of midbrain (arrow), OTec, and cerebellum, i) NH (arrow) and cortex of cerebellum, j) mucosal layer of anterior segment of gastrointestinal tract, k) NIL region of pituitary (arrow), l) serial section of k demonstrating total saturation of ^{125}I-[Tyr-4]-BBS-14 binding sites in the presence of 10^{-6}M BBS. Refer to Table 7-1 for nomenclature of abbreviated brain nuclei areas. **b)** Representative atlas drawings of the goldfish forebrain indicating nuclear regions which are described in A) as containing ^{125}I-[Tyr-4]-BBS-14 binding sites (after Peter and Gill, 1975).


a
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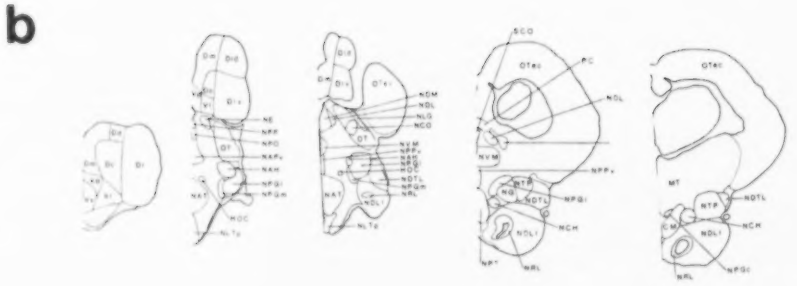
b

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Chapter 8

Preliminary Studies on Cholecystokinin Binding Sites in the Goldfish Central Nervous System

8.1 Introduction

In the goldfish, cholecystokinin (CCK) /gastrin-like immunoreactive (IR) peptides are widely distributed throughout the brain and anterior pituitary (Himick *et al.*, 1993; Himick and Peter, 1994; Chapter 5 and Chapter 6). Furthermore, *in vivo* and *in vitro* experiments in goldfish demonstrate that CCK/gastrin-like peptides are involved in the acute regulation of feeding behavior (Himick and Peter, 1994) and the regulation of pituitary growth hormone and gonadotropin release (Himick *et al.*, 1993). Although members of the CCK/gastrin family of peptides are biologically active within the goldfish, the sites of action where these peptides mediate their effects in the central nervous system remain unknown.

In mammals, studies characterizing the CCK receptor have reported two classes of CCK/gastrin binding sites (Sankaran *et al.*, 1980; 1982; for review Chapter 1). The first class is the CCK-B (brain)/gastrin receptor subtype, which interacts nearly equally well with all forms of sulfated and nonsulfated CCK and gastrin, and exhibits a widespread distribution throughout the central nervous system (for review Vigna, 1994). CCK-B receptors are distributed in the limbic, olfactory, visual and cortical areas (Saito *et al.*, 1980; for review Innis *et al.*, 1984). CCK receptors have also been described in the cerebellum of the guinea pig (Williams *et al.*, 1986; Niehoff, 1989). The second class of mammalian CCK binding sites is the CCK-A (alimentary) receptor subtype, which has a strict requirement for CCK peptides containing a sulfated tyrosine residue at position seven from the carboxyl terminus (for review Vigna, 1994). CCK-A receptors occur within organs and endocrine cells associated with the gastrointestinal tract, such as pancreatic acinar cells, pancreatic membranes, and gallbladder muscle (Sankaran *et al.*, 1980; Innis and Snyder, 1980; Morini *et al.*, 1990), but also in highly localized brain regions, and in the vagus nerve (Innis *et al.*, 1984; Moran *et al.*, 1986). Both CCK-A

and CCK-B receptors may have originated from a single "primitive" CCK (CCK-P) receptor which may still be present in lower ectothermic vertebrates (Vigna, 1994). The CCK-P binding site interacts with high affinity only with sulfated forms of CCK and gastrin (Vigna *et al.*, 1986).

To date, only one study has described the presence of high affinity, CCK/gastrin binding sites in fish. Using autoradiography, Moons *et al.* (1992) reported specific binding sites for [^{3}H]-CCK8-s in the brain of the sea bass *Dicentrarchus labrax*. High densities of CCK-like binding sites were described in the dorsal and ventral telencephalon and within the dorsal medulla oblongata just ventral to the vagal lobe, while lower densities of radiolabeled CCK8-s were localized in the anterior, tuberal, lateral, and posterior hypothalamus, in optic tectum, the valvula cerebelli, and vagal lobes.

Preliminary studies in this chapter have examined the presence and distribution of CCK/gastrin binding sites in the central nervous system of the goldfish. Data presented here indicate that binding sites for ^{125}I-Bolton Hunter-CCK8-s (^{125}I-BH-CCK8-s) are present in the hypothalamic feeding area of the goldfish, providing additional evidence for a role of CCK/gastrin-like peptides in the central control of food intake in fish. Furthermore, ^{125}I-BH-CCK8-s binding sites within the pars distalis of the pituitary indicate that the effects of CCK/gastrin-like peptides on pituitary growth hormone and gonadotropin release (Himick *et al.*, 1993) are likely mediated through direct actions at the level of the goldfish pituitary CCK/gastrin binding sites.

8.2 Materials and Methods

Experimental Animals

Male and female goldfish (25-45 g) were purchased from Ozark Fisheries (Stoutland, MO) at several times of the year. Fish were maintained under simulated Edmonton natural photoperiod in 1.8 kL tanks which received a constant flow of aerated and dechlorinated city water at 17^{o}C. Fish were fed *ad libitum* twice daily with commercially prepared fish pellets until used for autoradiographic studies.

Tissue Preparation

Brain and pituitary tissues of anesthetized goldfish were quickly dissected and rinsed

with ice-cold 0.1 M phospate buffered saline (PBS; pH 7.4). Tissues were immediately placed in Tissue-Tec embedding medium (Miles Inc, Elkhart, IN) and frozen on dry ice. Tissues were serially sectioned at 20 µm on a cryostat at -17°C, thaw-mounted on microscope slides (Superfrost/Plus, Fisher Scientific), and stored in sealed slide boxes containing drierite ($CaSO_4$) at -20°C until use.

Autoradiographic Technique

The autoradiographic technique used for localization of goldfish CCK/gastrin binding sites has been described previously by Niehoff (1988). Briefly, slides containing tissue sections were allowed to thaw to room temperature in a sealed slide box containing drierite and were then placed under vacuum for approximately thirty minutes. This later step enhanced tissue adherence to the slides, which was a problem due to the high lipid content of the unfixed brain tissues. The slide-mounted tissue sections were then preincubated for 30 minutes at room temperature in 50 mM Tris HCl, pH 7.4, containing 130 mM NaCl, 4.7 mM KCl, 5 mM $MgCl_2$, and 1 mM EGTA (= Tris saline buffer) plus 0.5% BSA. Sections were then incubated for 150 minutes at room temperature in Tris saline buffer (pH 6.5) containing 0.025% bacitracin, 1mM dithiothreitol, 2 µg/mL chymostatin, 4 µg/mL leupeptin, and ^{125}I-BH-CCK8-s (Dupont, Wilmington, DE) in the presence (control) or absence of 1 µM CCK for 60 minutes at room temperature. Conditions used in these binding experiments were based on preliminary studies which indicated that incubation of sections with radiolabeled CCK for 150 minutes at room temperature resulted in excellent ^{125}I-BH-CCK8-s binding, with very little non-specific binding. The slide-mounted tissue sections were then washed six times for 15 minutes each in Tris saline buffer containing 0.5% BSA at 4°C. Finally, the slides were rinsed twice for 5 seconds each at 4°C in distilled and deionized water. The slides were then dried at 4°C under a stream of cold air.

To visualize the localization of CCK receptors, the dried slides were placed in apposition to ß-Max High Performance Autoradiography Hyperfilm (Amersham, Oakville, ON) at room temperature for approximately 10 days. To eventually quantify the densities of binding sites in localized brain and pituitary areas on the autoradiograms, a ^{125}I-microscale (20 µM; Amersham, Oakville, ON) was also exposed to the film. The

film was then developed using Kodak D-19 Developer and fixed in undiluted Kodak Fixer. The film was washed under running tap water and hanged to dry. To identify brain nuclei, sections were stained with cresyl violet following exposure to autoradiography film.

Materials

^{125}I-BH-CCK8-s was obtained from Dupont, Wilmington, DE. Sulfated CCK8 (CCK8-s), nonsulfated CCK8 (CCK8-ns), gastrin II sulfated (GII-s) and nonsulfated gastrin I (GI-ns) were purchased from Bachem California (Torrance, CA).

Competitive Binding Studies

To determine the specificity of the CCK/gastrin binding site in the goldfish central nervous system, preliminary competitive binding experiments were conducted to examine the ability of CCK/gastrin related peptides to displace ^{125}I-BH-CCK8-s. Serial brain and pituitary sections were thaw-mounted on slides and then, following the above described autoradiographic technique, sections were incubated for 150 minutes at room temperature with a constant concentration of ^{125}I-BH-CCK8-s in the presence of one dose of 10^{-11} M to 10^{-6} M of the CCK/gastrin-related peptides: CCK8-s, CCK8-ns, GII-s, and GI-ns. Once the sections were washed and subsequently dried under a stream of cold air, they were placed in apposition to ß-Max High Performance Autoradiography Hyperfilm.

Autoradiographic Analyses

In these preliminary experiments, only the presence and distribution of ^{125}I-BH-CCK8-s binding sites were examined in the goldfish central nervous system. The use of ^{125}I-microscales, which were placed in apposition to the ß-Max High Performance Autoradiography Film at the same time as were the experimental slides, will allow future quantification of radiolabeled CCK binding in localized brain and pituitary regions through optical densitometric analyses. This will also allow me to eventually calculate the binding affinities of CCK/gastrin-related peptides, through construction of competitive binding curves based on actual densities of specific CCK radiolabel binding.

To determine the specificity of the CCK/gastrin binding site in the goldfish brain and

pituitary, visual analysis of the displacement abilities of several CCK/gastrin-related peptides were conducted over the dose range of 10^{-11}M to 10^{-6}M. The dose at which partial and full displacement of ^{125}I-BH-CCK8-s binding occurred was determined, and from these data the affinity of the binding site for that particular peptide could be estimated.

To visually quantify the density of ^{125}I-BH-CCK8-s in localized goldfish brain and pituitary regions, a ranking system was used whereby greatest density (++++) to lowest density (+) of binding sites was estimated by visually inspecting autoradiographs of sections which had been incubated with radiolabeled CCK8-s.

8.3 Results

Goldfish Pituitary

Preliminary studies indicated localization of ^{125}I-BH-CCK8-s binding sites within the pars distalis of the goldfish pituitary (Fig. 8-1 a, arrows; Table 8-1). When serial sections of the pituitary were incubated in varying doses of CCK8-s (10^{-6}M to 10^{-11}M) in the presence of ^{125}I-BH-CCK8-s, full displacement of radiolabeled CCK was observed at 10^{-9}M CCK8-s. Results from the competitive binding experiments indicated that full displacement of ^{125}I-BH-CCK8-s also occurred with 10^{-9}M GII-s. Only partial binding displacement of radiolabeled CCK8-s was detected when CCK8-ns and GI-ns were present at 10^{-7}M. Full displacement of ^{125}I-BH-CCK8-s occurred when CCK8-ns and GI-ns were present at 10^{-6}M.

Non-specific binding was negligible, as determined by the addition of 10^{-6}M CCK8-s in the presence of ^{125}I-BH-CCK8-s. These preliminary studies provide evidence that the CCK/gastrin-binding site in the goldfish pituitary is saturable and of high affinity for the sulfated forms of CCK and gastrin.

Goldfish Brain

When serial sections of brain regions were incubated with varying concentrations of CCK8-s (10^{-6}M to 10^{-11}M) in the presence of ^{125}I-BH-CCK8-s, full displacement of radiolabeled CCK8-s was evident at 10^{-9}M CCK8-s. Similarly, when data from the competitive binding studies of CCK/gastrin-related peptides were examined, 10^{-9}M

Table 8-1. Pituitary and brain nuclear areas containing ^{125}I-BH-CCK8-s binding sites and their relative densities (note: only brain areas which could be identified are listed).

Brain Area		^{125}I-BH-CCK8-s Binding Density
Diencephalon:		
Olfactory Areas and Telencephalon		
OB	olfactory bulb (distal end/olfactory tract)	++++
Vd	area ventralis telencephali pars dorsalis	+++
Vv	area ventralis telencephali pars ventralis	+++
Diencephalon		
Hypothalamus		
NAT	nucleus anterior tuberis	+++
NDLI	nucleus diffusus lobi inferioris	+++ (a) /++++ (p)
NDTL	nucleus diffusus tori lateralis	++
NG	nucleus glomerulosus	++
NH	nucleus habenularis	++
NLT	nucleus lateral tuberis	++
NPGL	nucleus preglomerulosus pars lateralis	++
NRL	nucleus recessus lateralis	++
NRP	nucleus recessus posterioris	++
Dorsal Diencephalon		
NVM	nucleus ventromedialis thalami	+++
Midbrain and Hindbrain		
OTec	optic tectum	++++
C	cerebellum	++
MT	midbrain tegmentum	+++
NIp	nucleus interpeduncularis	++
TS	torus semicircularis	++
VL	vagal lobe	++
FL	facial lobe	++
Pituitary		
PD	pars distalis	++++

+ = lowest binding/++++ = highest binding observed; (a) = anterior, (p) = posterior (nomenclature of brain nuclei after Peter and Gill, 1975).

GII-s produced full displacement of ^{125}I-BH-CCK8-s from brain sections. On the contrary, CCK8-ns and GI-ns only partially displaced CCK8-s radiolabeled binding from sections at 10^{-7}M, while full displacement of ^{125}I-BH-CCK8-s binding by these two peptides occurred at 10^{-6}M.

Non-specific binding by ^{125}I-BH-CCK8-s to sections was negligible, as determined by the addition of 10^{-6}M CCK8-s in the presence of radiolabeled CCK8-s. These preliminary studies provide evidence that, like in the pituitary, the CCK/gastrin-binding site in the goldfish brain is specific, saturable and of high affinity for the sulfated forms of CCK and gastrin.

Studies indicated that specific binding sites for ^{125}I-BH-CCK8-s were localized throughout the goldfish forebrain, midbrain and hindbrain (Table 8-1). Specific radiolabeled CCK8-s binding was detected in the distal end of the olfactory bulb (OB), and olfactory tracts (Fig. 8-1 b, arrow). Specific binding of CCK8-s was also localized in areas of the telencephalon, including the area ventralis telencephali pars ventralis (Vv), and the area ventralis telencephali pars dorsalis (Vd) (Fig. 8-1 c).

Specific binding sites for ^{125}I-BH-CCK8-s were also detected in areas of the ventro-posterior hypothalamus and within the hypothalamic inferior lobes, including the nucleus diffusus lobi inferioris (NDLI), nucleus recessus lateralis (NRL), nucleus lateral tuberis (NLT), nucleus anterior tuberis (NAT), nucleus preglomerulosus pars lateralis (NPGl), nucleus glomerulosus (NG), and nucleus diffusus tori lateralis (NDTL) (Fig. 8 e, Table 8-1). Additionally, dorsal brain regions also contained specific binding sites, including the optic tectum (OTec) (Fig. 8-1 d and e), the nucleus habenularis (NH), and several medial thalamic areas, such as the nucleus ventromedialis thalami (NVM) (Fig. 8-1 d and e; Table 8-1). Specific radiolabeled CCK binding was also detected in the cerebellum (Fig. 8-1 e).

In the central midbrain region, specific ^{125}I-BH-CCK8-s binding sites were localized in the nucleus interpeduncularis (NIp), the torus semicircularis (TS), and the midbrain tegmentum (MT) (Fig. 8-1 e). Finally, in the medulla of the hindbrain, the vagal lobe (VL) and the facial lobe (FL) also contained specific radiolabeled CCK8-s binding sites (Fig. 8-1 f, arrow = FL).

8.4 Discussion

When results from the competitive binding experiments were analysed for both brain and pituitary sections, ^{125}I-BH-CCK8-s binding was found to be fully displaced by the sulfated forms of CCK and gastrin. Radiolabeled CCK8-s binding in both the brain and pituitary was found to be partially displaced in the presence of 10^{-10}M CCK8-s or GII-s, and fully displaced in the presence of 10^{-9}M CCK8-s or GII-s. On the contrary, ^{125}I-BH-CCK8-s binding was only partially displaced in the presence of 10^{-7}M CCK8-ns or GI-ns, and fully displaced when these two peptides were present at 10^{-6}M, indicating that the CCK/gastrin binding site in the goldfish central nervous system exhibits an approximate 100- to 1000- fold greater affinity for the sulfated forms of CCK and gastrin, relative to the nonsulfated forms of CCK and gastrin. These preliminary studies also demonstrate that the ^{125}I-BH-CCK8-s binding sites in the goldfish brain and pituitary are specific for CCK/gastrin peptides. Such findings suggest that the CCK/gastrin binding site in fish does not have a strict requirement for the postitioning of the sulfated tyrosine residue (ie., CCK peptides: position 7 from the carboxyl terminus; gastrin peptides: position 6 from the carboxyl terminus). However, sulfation is required for high affinity binding.

Brain areas which contained ^{125}I-BH-CCK8-s specific binding sites included the base of the OB and olfactory tracts, the ventro-medial areas of the telencephalon, the ventro-posterior regions of the hypothalamus (including the hypothalamic inferior lobes), the OTec, several medial thalamic nuclei, and the midbrain tegmental areas (refer to Table 8-1 for binding in specific nuclear areas). In addition, ^{125}I-BH-CCK8-s specific binding sites were localized in the pars distalis of the anterior pituitary.

Preliminary findings presented here provide strong evidence for the existence of a primitive CCK (CCK-P) receptor at the level of the goldfish. This concept was recently proposed by Vigna (1994), who suggested that the CCK-P receptor gave rise to the two distinctive classes of CCK/gastrin receptors present in mammals, CCK-A and CCK-B (for review Chapter 1). This is believed to have occurred at the evolutionary level of divergence of endotherms and ectotherms. Findings of high affinity binding of the sulfated forms of CCK/gastrin peptides are also in agreement with *in vivo* and *in vitro* biological actions in several fish species which demonstrate increased efficacy with the sulfated forms of CCK or gastrin relative to the effects observed with the nonsulfated

forms (Vigna and Gorbman, 1977; Aldman and Holmgren 1987; Himick *et al.*, 1993)

Only one other study has reported the presence of CCK specific binding sites in fish. In *D. labrax*, Moons *et al.* (1992) reported specific binding sites for [^{3}H]-CCK8-s in the dorsal and ventral telencephalon, the preoptic, tuberal, and posterior hypothalamus, OTec, and the valvulla cerebelli. Specific binding was also detected in the dorsal medulla oblongata just ventral to the VL at the level of the facial and vagal motor nuclei. However, there was no evidence for the presence of CCK/gastrin binding sites in the sea bass pituitary, yet immunocytochemical studies on the teleost pituitary argues in favor of a hypophysial site of action of CCK (Moons *et al.*, 1988; Himick *et al.*, 1993, Chapter 6). In the present studies, ^{125}I-BH-CCK8-s specific binding sites were clearly present throughout the anterior goldfish pituitary. Localization of these ^{125}I-BH-CCK8-s binding sites parallel findings of a dense network of CCK/gastrin-like IR fibres also reported within the proximal pars distalis amongst both the gonadotropes and somatotropes (Himick *et al.*, 1993, Chapter 6). The presence of ^{125}I-BH-CCK8 binding sites in the goldfish anterior pituitary, together with the presence of CCK/gastrin-like IR distributed throughout the proximal pars distalis, provides evidence for a physiological role of CCK/gastrin peptides in the regulation of anterior pituitary hormone release in goldfish.

The presence of ^{125}I-BH-CCK8-s specific binding sites, as well as the localization of CCK/gastrin-like IR material (Himick and Peter, 1994; Chapter 6), within the hypothalamic feeding area (for review Demski 1981; 1983; Chapter 1) supports the hypothesis that CCK/gastrin-like peptides are involved in the regulation of feeding behavior in goldfish. Further evidence for a role of CCK in the control of food intake in goldfish is based on injection studies of CCK8-s either intraperitoneally or into the third brain ventricle, both of which cause an acute suppression in feeding behavior (Himick and Peter, 1994; Chapter 5). The presence of high concentrations of CCK/gastrin-like IR in the ventro-posterior hypothalamus and hypothalamic inferior lobes of several fish species, including the rainbow trout, *Oncorhynchus mykiss* (Notenboom *et al.*, 1981), the sea bass, *D. labrax* (Moons *et al.*, 1992), the green molly, *Poecillia latipinna* (Batten *et al.*, 1990) and in the goldfish *Carassius auratus* (Himick and Peter, 1994), as well as the presence of high affinity, specific CCK/gastrin binding sites in these brain

areas (Moons *et al.*, 1992; present study), and the *in vivo* effects of CCK8-s administration on food intake in goldfish (Himick and Peter, 1994, Chapter 5), all provide evidence that, besides having well established effects on feeding suppression in mammals (for reviews Morley, 1987; Silver and Morley, 1991), CCK/gastrin-like peptides also exert parallel effects with respect to the regulation of feeding behavior in lower vertebrates.

Radiolabeled CCK8-s binding was also detected in goldfish brain areas which are associated with the reception of external stimuli. The binding of endogenous CCK/gastrin peptides to these specific brain areas may be one mechanism whereby feeding stimuli are received from the environment and transmitted to the hypothalamic feeding center in fish. For example, ^{125}I-BH-CCK8-s binding sites were detected in the base of the OB and olfactory tract, as well as within the OTec, both of which represent main sensory areas for the detection of chemical and visual stimuli in the aqueous environment. Additionally, radiolabeled CCK binding was detected in the VL and FL of the medulla of the goldfish hindbrain, both of which are associated with the intraoral and extraoral taste systems, respectively. For example, the VL receives innervation from the vagus nerve which, in turn, innervates the oropharyngeal cavity and palatine organ. The main role of the VL is to perform discriminative sensorimotor functions leading to the selective ingestion of food (Kanwal and Finger, 1992). Conversely, the FL is innervated by the facial nerve which, in turn, innervates the outer skin surface and lips. Sensory receptors associated with the facial nerve can detect chemical stimuli at a distance and can subserve a food-localization function during food search (Kanwal and Finger, 1992). Together these brain areas transmit sensory information regarding the feeding environment to the hypothalamic feeding center and food intake is altered accordingly (for review Chapter 1). It is hypothesized that endogenous CCK/gastrin-like peptides, of either central origin and/or also from the peripheral circulation, bind to CCK8-s receptors located in the gustatory system located in the goldfish medulla.

Overall, preliminary studies in this chapter demonstrate that ^{125}I-BH-CCK8-s specific binding sites are present throughout the goldfish brain and anterior pituitary. Within the brain, CCK binding is present in the base of the OB and olfactory tracts, the ventro-medial areas of the telencephalon, the ventro-posterior regions of the hypothalamus and the hypothalamic inferior lobes, the OTec, and the anterior pituitary.

Based on preliminary competitive binding studies with several CCK/gastrin-like peptides, the ^{125}I-BH-CCK8-s binding site in the goldfish central nervous system has an approximate 100- to 1000- fold higher affinity for CCK/gastrin peptides which contain a sulfated tyrosine residue at position six or seven from the carboxyl terminus. The specificity of this binding site for sulfated CCK/gastrin peptides is also demonstrated by the partial and full displacement of radiolabeled CCK8-s by low amounts of CCK8-s or GII-s. The localization of binding sites within the hypothalamic feeding area indicates that centrally administered CCK8-s (Himick and Peter, 1994) likely acts within this brain region to suppress feeding behavior. In addition, the distribution of ^{125}I-BH-CCK8-s binding sites in areas associated with the input of external sensory information, such as the OB, OTec, and the FL and VL, suggests that members of the CCK/gastrin family of peptides may also play a role in the transmission of external feeding stimuli to the hypothalamic feeding center in fish where feeding behavior is subsequently regulated.

Fig. 8-1. Binding sites for ^{125}I-BH-CCK8-s in the goldfish brain and pituitary. Representative brain and pituitary sections from the goldfish which demonstrate ^{125}I-BH-CCK8-s binding sites in : a) anterior pituitary (arrows), b) OB and olfactory tract (arrow), c) Vv (arrow) and Vd of the telencephalon, d) OTec, NVM regions of the dorsal diencephalon, e) OTec, cerebellum, MT, NDLI, and several ventro-posterior hypothalamic nuclei, f) VL (outer layer) and FL (arrow). Refer to Table 8-1 for nomenclature of abbreviated brain nuclei areas.

a
b
c
d
e
f

8.5 References

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Chapter 9

Research Summary and General Discussion

Experiments conducted throughout this thesis provide evidence to support the hypothesis that cholecystokinin (CCK)/gastrin-like peptides and bombesin (BBS)/gastrin-releasing peptides (GRP) play a role in the peripheral and central regulation of feeding behavior in goldfish. Furthermore, these peptides may also act in fish as physiological regulators of anterior pituitary hormone release. These data are unique in that they are the first studies to document in detail the effects of a neuropeptide and its receptor binding sites on the alteration of food intake within any lower vertebrate. Below is a brief summary of the data contained within this thesis. This chapter is concluded by an overall general discussion based on the interpretation of findings presented herein.

9.1 Research Summary

i) Distribution of CCK/gastrin-Like and BBS/GRP-Like Peptides in the Goldfish Central Nervous System

Based on immunohistochemical studies using antisera specific to the carboxyl-terminal tetrapeptide sequence of CCK/gastrin, CCK/gastrin-like immunoreactivity (IR) was extensively distributed throughout ventral regions of the goldfish brain, ranging from the telencephalon, through the hypothalamus and thalamus, within the midbrain tegmentum, and in the vagal lobes and facial lobe of the hindbrain. The highest concentration of CCK/gastrin-like IR material was consistently found within fibres and cell bodies of the hypothalamic inferior lobe and more ventro-posterior regions of the hypothalamus, including the nucleus preglomerulosus (NPGl), nucleus glomerulosus (NGl), nucleus anterior tuberis (NAT), nucleus lateralis tuberis (NLT), nucleus recessus lateralis (NRL), nucleus recessus posterioris (NRP), and nucleus posterioris periventricularis (NPPv). Other brain regions which contained a high degree of CCK/gastrin-like IR included areas within the preoptic hypothalamus, such as the nucleus entopeduncularis (NE), nucleus preopticus periventricularis (NPP), and nucleus preopticus (NPO), as well as regions within the dorsal diencephalon and midbrain, such

as the nucleus habenularis (NH), optic tectum (OTec), and several thalamic nuclei.

Within the goldfish pituitary, intense CCK/gastrin-like IR was consistently detected in fibres of the proximal pars distalis (PD), with fewer IR fibres localized in the rostral PD. In the proximal PD, CCK/gastrin-like IR fibres were distributed amongst both the gonadotropes and somatotropes, suggesting a possible role for CCK-like peptides in the regulation of gonadotropin-II (GtH-II) and growth hormone (GH) secretion.

CCK/gastrin-like IR was also localized within the gut of the goldfish. Only the anterior one-third of the gastrointestinal tract was examined; however, CCK/gastrin-like IR material was detected within endocrine cells of the mucosal layer and in nerve fibres of the submucosal and circular muscle layers.

Using antisera specific to the carboxyl terminal of synthetic BBS-14, BBS/GRP-like IR fibres were localized in the ventral forebrain. In the preoptic hypothalamus, BBS/GRP-like IR fibres were identified within the NE, NPP, and the NPO, while in more ventro-posterior hypothalamic regions BBS/GRP-like IR material was detected in the NLT, NAT, NRL, and NRP, and NDLI. Perikarya containing BBS/GRP-like IR were rarely seen (even following brain ventricle colchicine injection), but occasionally detected in several periventricular regions of the ventro-posterior hypothalamus. BBS/GRP-like IR fibres and few IR perikarya were also localized within dorsal regions of the diencephalon, including the nucleus rotundus (NR) and several thalamic nuclei, as well as within the nucleus interpeduncularis (NIp), and OTec of the midbrain.

BBS/GRP-like IR fibres were consistently detected throughout the neurointermediate lobe (NIL) of the goldfish pituitary. Occasionally, cells within the PD of the anterior pituitary also contained BBS/GRP-like IR. BBS/GRP-like IR was also detected in endocrine cells of the mucosal layer in the anterior one-third of the goldfish gastrointestinal tract.

ii) CCK and BBS Act to Suppress Feeding Behavior in Goldfish

The widespread distribution of CCK/gastrin-like IR and BBS/GRP-like IR material in the goldfish gut and brain, and in particular within the ventro-posterior hypothalamus and the hypothalamic inferior lobes (or the proposed teleost brain "feeding center") suggests that CCK and BBS may be involved in the regulation of feeding behavior in fish. This hypothesis was confirmed when CCK8-s and BBS were injected either intraperitoneally

(ip) or into the third brain ventricle (icv) of goldfish and the result was an acute suppression of food intake during 45 minutes post-administration. When a single dose of 50 or 500 ng/g CCK8-s was ip injected into fish, food intake was reduced 54% and 81%, respectively. When injected icv with a high dose of CCK8-s (50 ng/g), feeding was decreased 95%, while icv injection of a 10-fold lower dose of CCK8-s resulted in a 50% suppression in food intake. The sulfated form of CCK8 was more effective than CCK8-ns in suppressing feeding behavior. Goldfish which received 100 ng/g CCK8-s decreased their food intake 58%, while fish ip injected with an equimolar dose of CCK8-ns exhibited only an 11% inhibition in feeding. Occasionally, changes were observed in circulating levels of serum GH levels following peripheral or third brain ventricle injection of CCK8-s. These changes were not consistent, however, and may be a reflection of the seasonality in the GH responsiveness to CCK peptides as observed *in vitro* following perifusion of goldfish pituitary fragments with CCK8-s.

Goldfish which were injected ip with increasing doses of BBS (0.5 ng/g - 100 ng/g) also exhibited a dose-dependent inhibition in food intake over 45 minutes of administration. Maximal suppression of 88% was achieved at 50 ng/g BBS, while 24 ng/g BBS was sufficient to inhibit food intake 68%. Associated with ip BBS injection was a "spitting out" behavior; food pellets were taken into the buccal cavity of fish, but immediately expelled. Goldfish which were deprived of food for 72 hours still exhibited an 80% decrease in food intake when injected with 50 ng/g BBS, indicating the potency of this neuropeptide to inhibit feeding in fish. Goldfish which were icv injected with 10 ng/g BBS also exhibited an 82% decrease in feeding activity. Accompanying both peripheral and icv injection of BBS and suppressed food intake were consistent elevations in circulating serum GH. When groups of goldfish were injected icv with 5 or 50 ng/g BBS, a graded increase in serum GH levels occurred with increasing peptide dosage at 45 minutes post-administration. Similarly, when groups of fish were injected ip with 10 or 100 ng/g BBS, a significant increase in circulating serum GH levels occurred at 1.5 hours post-injection.

iii) Neuroendocrine Actions of CCK/gastrin- and BBS/GRP-Like Peptides in the Goldfish Pituitary

Based on the extensive distribution of CCK/gastrin-like IR and BBS/GRP-like IR within the goldfish pituitary, it is likely that both peptides are involved in the regulation of pituitary hormone release. Studies within this thesis indicate that both CCK/gastrin-like peptides and BBS/GRP-like peptides are capable of directly acting at the level of the goldfish pituitary to stimulate the release of GH and GtH-II *in vitro*.

Exposure of pituitary fragments from either gonadal recrudescing (maturing) or regressed goldfish to three 5 minute pulses of 1.0 or 10 nM CCK8-s produced an increase in the secretion of both GtH-II and GH, while repeated challenge of pituitary fragments to 10 nM CCK8-s resulted in a desensitization in the GH release response to the second or the third pulse. Other studies have indicated that fragments from sexually regressed goldfish exhibit a greater release response of GtH-II to CCK8-s relative to fragments from sexually recrudescing fish. The GH release responses to CCK8-s were similar between the two sexual stages, however. Additionally, CCK8-s and gastrin 17-sulfated (G17-s) exhibited greater stimulatory abilities than CCK8-ns in releasing GtH-II and GH from fragments of sexually recrudescing fish, indicating the importance of tyrosine sulfation for enhanced activation of the CCK/gastrin receptor within the goldfish pituitary. CCK8-s and G17-s were equal in their capacity to stimulate the release of GtH-II, confirming the proposal by Vigna (1994) that the CCK/gastrin receptor in fish is primitive (CCK-P) in that it does not appear to have any strict requirement for the positioning of the sulfated tyrosine residue (i.e., CCK: position seven from the carboxyl terminus; gastrin: position six from the carboxyl terminus). This is unlike the two distinct endothermic CCK receptors (CCK-A and CCK-B), which are present in endotherms and which have strict requirements for both the presence and location of a sulfated tyrosine. Findings of a co-distribution of CCK/gastrin-like IR amongst the gonadotropes and somatotropes of the proximal PD, and the *in vitro* release of both GH and GtH-II from goldfish pituitary fragments following perifusion of CCK8-s, indicates that members of the CCK/gastrin family of peptides play a physiological role in the regulation of anterior pituitary hormone release in fish.

BBS/GRP-like IR was localized predominantly within fibres of the goldfish NIL,

although BBS/GRP-like IR material was occasionally detected in cells of the PD. When examined *in vitro* using the goldfish perifusion system, BBS/GRP-like peptides were capable of releasing both GH and GtH-II from pituitary fragments. The responsiveness of GH and GtH-II release to BBS challenge was variable; no indication of a clear dose-dependent effect of BBS on hormone release was evident, and the GH and GtH-II responsiveness to BBS was inconsistent throughout the season. However, perifusion of goldfish pituitary fragments with initial pulses of BBS (0.1, 1.0, 10, or 1000 nM) stimulated both GH and GtH-II release. While repeated challenge of 0.1 nM BBS produced a sensitization in the GH and GtH-II release responses, repeated pulses of 100 nM BBS from fragments of gonadal regressed fish resulted in desensitized GH and GtH-II release responses. Several BBS-like peptides, including neuromedin C (GRP-10), GRP 1-27, GRP 14-27 and neuromedin B also stimulated GH and GtH-II secretion.

iv) CCK/gastrin and BBS/GRP Receptor Binding Sites in the Goldfish Central Nervous System

Preliminary autoradiography experiments in this thesis demonstrated that high affinity, specific binding sites for ^{125}I-BH-CCK8-s were present in the goldfish central nervous system. Brain areas which appeared to contain the richest source of radiolabeled CCK8-s specific binding sites were the olfactory bulb and olfactory tracts, nuclei within the ventro-medial areas of the telencephalon and ventro-posterior hypothalamus (including the hypothalamic inferior lobes), the OTec, several medial thalamic nuclei, and the midbrain tegmentum. Specific radiolabeled CCK8-s binding was also detected in the PD of the anterior pituitary.

In both the brain and pituitary, the ^{125}I-BH-CCK8-s binding site was specific and of higher affinity for the sulfated forms of CCK and gastrin peptides, as opposed to the nonsulfated forms. Preliminary data on competitive binding curves of several related CCK/gastrin-like peptides also revealed that displacement of ^{125}I-BH-CCK8-s binding was approximately 100 to 1000 fold lower in the presence of the nonsulfated forms of CCK and gastrin, indicating that the CCK/gastrin binding site in the goldfish central nervous system has a strict requirement for only the presence of a sulfated tyrosine residue, and not for the positioning of this sulfated residue within the CCK/gastrin

sequence. These findings support the existence of a primitive CCK (CCK-P) receptor at the evolutionary level of fish as has been proposed by Vigna (1994).

Specific binding sites for ^{125}I-[Tyr-4]-BBS-14 were also localized in the goldfish central nervous system. BBS specific binding was detected in several brain areas including the olfactory bulb and nerve, the medial and dorso-lateral telencephalon, nuclei within the ventro-posterior hypothalamus (including the inferior lobes), the OTec, and the cortex of the cerebellum. In addition, specific binding of ^{125}I-[Tyr-4]-BBS-14 was demonstrated in the NIL of the goldfish pituitary, while low radiolabeled BBS binding was also detected in the PD.

In both the brain and pituitary, preliminary experiments indicated that the binding sites for ^{125}I-[Tyr-4]-BBS-14 were specific, and of higher affinity for the BBS/GRP-like peptides BBS-14, GRP 1-27, and neuromedin C, as opposed to neuromedin B and the BBS fragment (8-14). Displacement of ^{125}I-[Tyr-4]-BBS-14 by neuromedin B and the BBS fragment (8-14) was approximately 10- to 100-fold lower than the other BBS/GRP-like peptides examined in the competitive binding curves. These data suggest that the BBS/GRP binding site in the goldfish central nervous system is similar to the BBS/GRP receptor present in mammals. A strict requirement for the BBS/GRP carboxyl terminal sequence of greater than seven amino acid residues is necessary for high affinity binding. Variations in this C-terminal sequence result in lower binding affinity.

9.2 General Discussion

Research findings presented in this thesis provide strong evidence for a role of CCK/gastrin-like and BBS/GRP-like peptides in the short-term regulation of feeding behavior in goldfish. While the actions of multiple neuropeptides on the central control of food intake and the hypothalamic feeding drive system have been well established in mammals (for review Morley, 1985), these are the first studies to document the effects of a neuropeptide in the central and peripheral control of food intake in a lower vertebrate. The regulation of feeding behavior is a complex event which, in mammals, involves interactions among many peptides, hormones, neurotransmitters, and humoral substances (for review Morley, 1985). Studies presented in this thesis have focused on only two neuropeptides and their influences on feeding behavior in the goldfish. However, it is hoped that these findings will provide a foundation for future research in this novel area

of fish physiology.

i) BBS and CCK Regulate Feeding Behavior in Goldfish: Central Effects on Food Intake

In mammals, it is well established that body weight is regulated to a "set-point" level, and that this is accomplished through the regulation of feeding behavior (Panksepp, 1971; 1974). Likewise, Peter (1979) proposed that fish regulate the amount of food they consume based on an internal "set-point", which subsequently results in the maintenance of body growth and weight. For example, early studies conducted by Rozin and Mayer (1961, 1964) reported that conditioned goldfish had distributed their feeding responses fairly evenly over the light phase. These fish demonstrated accurate regulation of the amount of food they consumed, which was ultimately based on caloric and nutrient content, as well as the ambient temperature. If these goldfish were presented with 20 mg pellets as opposed to 45 mg pellets, they consumed more food to satisfy their appetite. Likewise, if fish were given a diluted form of a kaolin diet, they consumed an increased amount of food to compensate for the dilution factor. With respect to influence of temperature on the regulation of feeding behavior, goldfish demonstrated a decrease in the amount of food they consumed by one half to one third, when water temperature was decreased by 10^{o}C. Other fish species have also demonstrated the capacity to regulate the amount of food they consume, including northern pike *Esox lucius* (Johnson, 1966), rainbow trout, *Oncorhynchus mykiss* (Lee and Putnam, 1973), and winter flounder, *Pseudopleuronectes americanus* (Tyler and Dunn, 1976). These studies demonstrate that like mammals, fish precisely regulate the amount of food they consume through a metabolic set-point established from the "milieu interieur".

As in mammals, an area within the teleost brain has been implicated in the neural regulation of feeding behavior, and this brain area has been localized within the ventro-posterior hypothalamus and the hypothalamic inferior lobes (for reviews Demski, 1981; 1983). Regions near the lateral recess of the third ventricle in the inferior lobe of the hypothalamus (nucleus recessus lateralis; NRL), or in areas just dorsal to the NRL and slightly below the nucleus preglomerulosus (NPGl), have been reported to alter feeding responses in tilapia, *Tilapia macrocephala* (Demski, 1973), and goldfish, *Carassius auratus* (Savage and Roberts, 1975). Electrical activation of the hypothalamic inferior

lobe has also been shown to alter feeding responses in the carp, *Cyprinus carpio* (Redgate, 1974). Together these data indicate that the ventro-posterior hypothalamus and the hypothalamic inferior lobes are important brain regions with respect to the control of feeding behavior in fish. The fact that manipulation of hypothalamic areas can alter feeding activity in fish suggests that neuropeptides are likely involved in the central control of food intake. Yet, studies directed towards this area of research have received little attention since the initial discovery of the fish hypothalamic feeding area, which was reported over 30 years ago. In mammals, the roles that neuropeptides play in the regulation of appetite have been well established (for reviews Morley, 1985; McCoy and Avery, 1990; Silver and Morley, 1991).

Data in this thesis have provided evidence to support the hypothesis that the neuropeptides CCK and BBS are involved in the central regulation of feeding behavior in the goldfish. Both CCK/gastrin-like IR and BBS/GRP-like IR material and specific, high affinity binding sites for both of these peptides are widely distributed throughout the ventro-posterior hypothalamus and hypothalamic inferior lobes (hypothalamic feeding area). Evidence for the presence of ^{125}I-[Tyr-4]-BBS-14 and ^{125}I-BH-CCK8-s binding sites within the NRL, NPGl and/or NG, and the NDLI of the ventro-posterior hypothalamus, together with the localization of BBS/GRP-like IR and CCK/gastrin-like IR material in these brain regions, indicates that BBS/GRP-like peptides and CCK/gastrin-like peptides and their respective binding sites are present in the same hypothalamic nuclei which participate in the control of food intake in fish. In fact, the presence of CCK/gastrin-like IR material within the hypothalamic feeding area of other teleost species, including *O. mykiss*, the sea bass (*Dicentrarchus labrax*), and the green molly (*Poecillia latipinna*), as well as the localization of specific, high affinity binding sites for CCK/gastrin in the ventro-posterior hypothalamus of *D. labrax*, indicates that CCK/gastrin-like actions within the brain to regulate feeding behavior, may be widespread throughout the teleosts (Notenboom *et al.*, 1981; Batten *et al.*, 1990; Moons *et al.*, 1992).

Studies in this thesis have shown that, in addition to the presence of BBS/GRP-like IR and CCK/gastrin-like IR peptides and ^{125}I-[Tyr-4]-BBS-14 and ^{125}I-BH-CCK8-s specific binding sites in the goldfish hypothalamic feeding area, icv injection of BBS-14 or CCK8-s results in the acute suppression of cumulative food intake. Intraventricular

injection of 50 ng/g CCK8-s or 60 ng/g BBS produced a 95% and 82% decrease in the amount of food consumed relative to saline injected controls, respectively. When a 10-fold lower dose of CCK8-s (5 ng/g) was injected, a 50% suppression in food intake occurred, suggesting that the effects of central CCK8-s administration on goldfish feeding behavior are dose-dependent. At present, it remains unknown where exogenously administered CCK8-s and BBS were acting in the goldfish brain to decrease food intake. In Chapters 3 and 5, it was speculated that injection of CCK8-s and BBS into the ventricular fluid system may reach periventricular sites within the ventro-posterior hypothalamus and hypothalamic inferior lobes, such as the NRL, NRP or NPPv. The findings of both ^{125}I-[Tyr-4]-BBS-14 and ^{125}I-BH-CCK8-s binding sites in these periventricular brain areas indicate that such an event may occur. It is hypothesized that during feeding in goldfish, endogenously released BBS/GRP-like and CCK/gastrin-like peptides bind to their specific, high affinity receptors located in the hypothalamic feeding area. This subsequently results in the initiation of satiation.

In mammals, the effects of central administration of BBS and CCK8-s on feeding behavior have been extensively investigated, and it has been reported that both peptides cause an immediate suppression in food intake by interacting with other neuropeptides and neurotransmitters located in the central feeding drive system of the lateral hypothalamus (for reviews see Morley, 1985; McCoy and Avery, 1990; Silver and Morley, 1991). Willis *et al.* (1984) described reduced food intake within 60 minutes of BBS injection into the paraventricular nucleus of the hypothalamus, while Stuckey and Gibbs (1982) reported decreased meal size within 30 minutes of bilateral BBS injection. Similarly, CCK8-s injection into the lateral ventricles of sheep causes a reduction of food intake within 30 minutes. In the dog, infusion of CCK8-s into the third cerebral ventricle also results in suppressed feeding. The fact that the biological actions of BBS and CCK are conserved between mammals and fish with respect to the regulation of feeding behavior, is likely a reflection of the highly conserved biologically active carboxyl termini sequences of the BBS and CCK molecules throughout vertebrate evolution.

Interestingly, other goldfish brain areas were also found to contain both BBS/GRP-like IR and CCK/gastrin-like IR material, and ^{125}I-[Tyr-4]-BBS-14 and ^{125}I-BH-CCK8-s specific binding sites. In particular, BBS/GRP-like and CCK/gastrin-like

peptides and their respective binding sites were localized in the vagal lobes and facial lobe in the medulla of the hindbrain. In fish, the gustatory system is divided into two subsystems; the facial taste system, which can detect chemical stimuli at a distance, and therefore subserves a food-localization function during food search, and the vagal taste subsystem, which performs a discriminitive sensorimotor function leading to the selective ingestion of food (Kanwal and Finger, 1992). In the goldfish, the well developed vagal lobes and relatively small facial lobe indicates that the vagal taste subsystem predominates, and that "selective ingestion" is the more critcial phase during feeding activity. The presence of both BBS/GRP-like IR and CCK/gastrin-like IR material and their binding sites in the goldfish vagal and facial lobes, suggests that BBS/GRP-like and CCK/gastrin-like peptides may participate in receiving incoming feeding-related sensory information based on selective ingestion, and subsequently transmit this information to the hypothalamic feeding center where feeding behavior is modified.

BBS/GRP-like and CCK/gastrin-like peptides may also participate in other brain areas to control food intake in the goldfish. For example, the OTec was shown to contain BBS/GRP-like IR and CCK/gastrin-like IR material and ^{125}I-[Tyr-4]-BBS-14 and ^{125}I-BH-CCK8-s binding sites. This well developed brain area in the goldfish receives sensory information from the eye via the optic tract, as well as from the olfactory bulb via the lateral olfactory tract which enters the optic nerve (Ariens Kappers *et al.*, 1965). In turn, fibres from the OTec project to various brain areas, including the cerebellum, the medulla, the thalamus and regions within the ventro-posterior hypothalamus. It is possible that endogenous BBS/GRP-like and CCK/gastrin-like peptides of the goldfish OTec are involved in the processing of incoming visual and olfactory sensory information which is associated with the searching and localization of a food source.

Areas within the telencephalon were also shown to contain BBS/GRP-like IR CCK/gastrin-like IR material. Binding sites for ^{125}I-[Tyr-4]-BBS-14 and ^{125}I-BH-CCK8-s were detected in the ventro-medial regions (^{125}I-BH-CCK8-s), and throughout the dorsal and ventral regions (^{125}I-[Tyr-4]-BBS-14) of the telencephalon. In fish, neuronal mapping studies have shown that the ventral portion of the area dorsalis pars medialis (Dm) and the medial region of the area dorsalis pars centralis (Dc) represent part of the gustatory system (Kanwal and Finger, 1992). Gustatory information is received in these telencephalic regions from the medulla of the hindbrain. In turn, this telencephalic

gustatory region sends projections into the NPGl and NRL of the hypothalamic feeding area (Kanwal and Finger, 1992). The presence of both BBS/GRP-like IR and CCK/gastrin-like IR peptides and their binding sites in this telencephalic gustatory region provides evidence that members of the BBS/GRP and CCK/gastrin family of peptides participate in the reception of gustatory sensory information outside of the hypothalamic feeding area in fish.

Restricted ventro-lateral regions and ventro-medial regions of the telencephalon have also been reported to receive input from the olfactory system. Since the presence of BBS/GRP-like IR and CCK/gastrin-like IR and/or their binding sites were found in the olfactory bulb, the olfactory nerve, or olfactory tracts, as well as within these restricted telencephalic areas, it remains possible that BBS/GRP-like and CCK/gastrin-like peptides also participate in the reception and transmission of olfactory sensory information from the telencephalon to the ventro-posterior hypothalamus during feeding. It has been reported by Stacey and Kyle (1983) that sectioning of the lateral olfactory tract reduces feeding behavior in the goldfish. The presence of ^{125}I-BH-CCK8-s binding sites in the goldfish olfactory tracts indicate that CCK/gastrin-like peptides are capable of acting at sites associated with the reception of feeding stimuli from outside of the hypothalamus. This olfactory information is then transmitted to the hypothalamic feeding area (Murakami *et al.*, 1983) where integration of incoming sensory information from all brain regions is processed and a feeding response is subsequently mediated.

ii) BBS and CCK Regulate Feeding Behavior in Goldfish: Peripheral Effects on Food Intake

Data in this thesis provide evidence that BBS/GRP-like IR and CCK/gastrin-like IR and their binding sites are also present outside of the goldfish central nervous system. BBS/GRP-like IR (data not shown) and CCK/gastrin-like IR (Chapter 5; Himick and Peter, 1994) were localized in both endocrine cells and within nerve fibres of the submucosa of the goldfish gut. In addition, ^{125}I-[Tyr-4]-BBS-14 (Chapter 7) and ^{125}I-BH-CCK8-s binding sites (data not shown) were detected in the gut, and preliminary evidence indicates that ^{125}I-[Tyr-4]-BBS-14 binding sites were also located on the goldfish gallbladder.

The presence of BBS/GRP-like IR and CCK/gastrin-like IR and their binding sites in

the goldfish gut indicates that these peptides are involved in the regulation of feeding activity. BBS/GRP-like IR and CCK/gastrin-like IR have been reported within the gut of other fish species, including the Atlantic cod *Gadus morhua* (Jonsson *et al.*, 1987; Holmgren and Jonsson, 1988), and the rainbow trout *O. mykiss* (Holmgren *et al.*, 1982; Bjenning and Holmgren, 1988), indicating a widespread role for BBS and CCK in the regulation of gastrointestinal functions. Furthermore, while no studies have examined the role of neuropeptides in the peripheral regulation of feeding behavior in fish, the effects of BBS and CCK8-s on feeding-related activities, such as the alteration in gut and gallbladder contractility (Vigna and Gorbman, 1977; Aldman and Holmgren, 1987; Jonsson *et al.*, 1987; Holmgren and Jonsson, 1988; Thorndyke and Holmgren, 1990), gastric acid secretion (Holstein, 1982), and gastric blood flow (Bjenning *et al.*, 1990) have been described.

Findings in this thesis indicated that when injected ip into goldfish, low doses of CCK8-s (50 ng/g or 500 ng/g) or BBS (0.5 ng/g to 50 ng/g) are effective in acutely suppressing cumulative food intake within 30 minutes. It was also shown that peripheral injection of the nonsulfated form of CCK8 did not cause a significant suppression in feeding behavior. Such findings can be explained through data obtained in Chapter 8, which demonstrate that the goldfish ^{125}I-BH-CCK8-s binding site has an approximate 100- to 1000-fold increased affinity for the sulfated forms of CCK and gastrin, as opposed to the nonsulfated forms of CCK and gastrin. In preliminary experiments, the effects of gastrin II-sulfated have also been examined on feeding behavior in the goldfish, and decreased feeding behavior has been observed. However, the effects of BBS/GRP-related peptides on feeding behavior in goldfish have not been investigated. Based on preliminary binding displacement studies of radiolabeled BBS by BBS/GRP-related peptides (Chapter 7) however, it is likely that BBS/GRP-like peptides containing the intact biologically active carboxyl terminal sequence of greater than 7 amino acids would also produce suppressed feeding activity in the goldfish.

One mechanism whereby peripherally administered BBS and CCK8-s may act to decrease cumulative food intake in goldfish is through the alteration of mechanical events associated with feeding. Both CCK8-s and BBS are capable of increasing the contractility of gastrointestinal longitudinal and circular muscles in fish (Aldman and

Holmgren, 1987; Jonsson *et al.*, 1987; Holmgren and Jonsson, 1988; Thorndyke and Holmgren, 1990; see discussion above). In the present studies (Chapter 3; Himick and Peter, 1994), it was demonstrated that associated with ip BBS injection and subsequent feeding suppression in goldfish was a second behavior which involved spitting out or expelling of pellets from the buccal cavity. Such effects may occur through the actions of peripherally administered BBS to the ^{125}I-[Tyr-4]-BBS-14 binding sites discovered in the goldfish gut (Chapter 7). This may then alter the mechanical events associated with the swallowing and/or digestion of food. Since CCK/gastrin-like peptides have also been shown to alter gut contractility (Jonnson *et al.*, 1987), such a mechanism may also apply to peripherally administered CCK8-s.

Alternatively, it is also possible that ip administered BBS and/or CCK8-s enters the blood circulation, and gains access to brain regions which are open to the blood brain barrier. For example, BBS and/or CCK8-s may come in contact with the ventral wall of the preoptic recess, which is located outside of the blood brain barrier (mammalian homologue = organum vasculosum laminae terminalis) (Peter *et al.*, 1980). Or BBS and/or CCK8-s may have access to ^{125}I-[Tyr-4]-BBS-14 and/or ^{125}I-BH-CCK8-s binding sites which have been localized in regions of either the nucleus lateralis tuberis (mammalian homologue = arcuate nucleus), the circumventricular organs, or regions of the hindbrain (Chapters 7 and 8), and which are all partially located outside the blood brain barrier (Peter *et al.*, 1980). If peripherally injected BBS or CCK8-s binds to areas associated with the vagal lobes, including the vagal nerve, then the "selectivity" or palatability of the food source may be altered, which may change feeding levels (refer to "Central effects of BBS and CCK on feeding").

In mammals, the effects of peripheral administration of CCK8-s and BBS on feeding suppression have been well studied. Initial studies by Gibbs *et al.* (1973) proposed that CCK acts as a peripheral satiety signal based on findings of suppressed solid and liquid food intake in rats following CCK8-s injection. CCK has since been shown to suppress feeding in a number of species following peripheral injection. In the cat, systemic infusion of CCK8-s has been reported to cause short-term transient satiation (Bado *et al.*, 1989) while in humans, intravenous CCK8-s administration results in a decrease in both solid and liquid food intake (Stacher, 1985). Raybould *et al.* (1988) have reported that peripheral CCK8-s acts to suppress feeding through CCK activation of vagal

mechanoreceptive endings in the gastric corpus wall; CCK-stimulated vagal afferents then input signals to central satiety centers via the nucleus tractus solitarius of the hindbrain. In support of this, Miceli (1985) has demonstrated that gastric vagotomy in hamsters results in blocked feeding suppression following systemic injection of low doses of CCK8-s.

Likewise, early studies by Gibbs *et al.* (1979) and Martin and Gibbs (1980), have reported that peripheral injection of BBS into rats elicits the normal sequence of behaviors associated with satiety, including grooming, sniffing, locomotion and rearing, followed by withdrawal from the food site, and rest or sleep. Other more recent studies have confirmed such findings. For example, peripheral injection of BBS into food-deprived rats results in a significant decrease in protein intake within 30 minutes (Avery and Livosky, 1986), while McCoy *et al.* (1990) similarly described a selective dose-dependent suppression in protein intake within 30 minutes following BBS injection. Such actions by peripherally administered BBS may occur, in part, through BBS-induced alterations in gastrointestinal motility and intragastric pressure (Falconiere *et al.* 1988, Margolis *et al.*, 1989) and/or altered stomach contractility (Deutsch, 1980).

The search for selective BBS and CCK antagonists which will be effective at the level of the goldfish BBS/GRP and CCK/gastrin receptor, has lead to preliminary *in vitro* studies investigating the effects of mammalian BBS antagonists on GH and GtH-II release from perifused goldfish pituitary fragments. Once a suitable antagonist is uncovered, the effects of this substance with respect to feeding behavior will be examined *in vivo* in goldfish. However, based on data presented in this thesis, it can be stated that the actions of both centrally and peripherally administered BBS and CCK8-s on feeding behavior in fish are specific. This is based on several observations. Firstly, it was reported that the nonsulfated form of CCK8 did not significantly reduce cumulative food intake levels in goldfish while CCK8-s was effective in decreasing feeding, indicating that actions mediated by CCK8-s are specific and that a sulfated tyrosine residue is required for CCK/gastrin-like peptide actions. Secondly, both CCK8-s and BBS effects on feeding suppression were dose-dependent in the goldfish, whether these peptides were administered centrally or peripherally. Finally, it was observed that following either icv or ip BBS or CCK8-s administration, goldfish were still active in searching for food

in their aquarium. Once the food source was located, the fish positioned themselves directly under the floating pellets and began to feed. There was no delay in the latency time from food administration to commencment of feeding, which indicated that BBS and CCK8-s did not cause malaise. However, while saline-injected fish consumed food at levels which were comparable to their feeding rates in an unhandled state, BBS and CCK8-s injected goldfish consumed overall less food within the experimental time period.

These observations provide strong support for a specific role of CCK/gastrin-like and BBS/GRP-like peptides in the central and peripheral regulation of feeding behavior in the goldfish. The proposed involvement of CCK/gastrin-like and BBS/GRP-like peptides in the control of food intake in the goldfish are illustrated in Fig. 9-1.

iii) Does a Relationship Exist Between BBS and CCK, Feeding Behavior, and Growth Hormone in Goldfish?

Experiments in Chapter 2 indicated that associated with food intake in unhandled goldfish were rapid and transient alterations in serum GH levels. Following administration of a restricted ration of 2% body weight, fish exhibited acute elevations in serum GH within 30 minutes of feeding. In a second phase, serum GH levels in fish decreased within 45 minutes to 60 minutes of feeding. This second phase of postprandial serum GH alterations was dependent on ration size; fish receiving either a 2% or 3% ration exhibited significantly lower GH levels relative to unfed controls or fish consuming only a 1% ration. Such postprandial changes in circulating GH levels may have been the result of humoral and/or neural influences at the level of the goldfish pituitary somatotropes.

In subsequent experiments in Chapter 3, it was reported that concomitant with decreased feeding following either icv or ip administration of BBS into goldfish were alterations in circulating levels of serum GH, suggesting that an interaction between BBS and GH may occur during feeding suppression. Experiments had indicated that following ip BBS administration of 10 ng/g or greater, serum GH levels were elevated from 45 minutes to at least 90 minutes post BBS-injection. On the contrary, at only 15 to 30 minutes following BBS injection, fish exhibited a decrease in serum GH levels. In

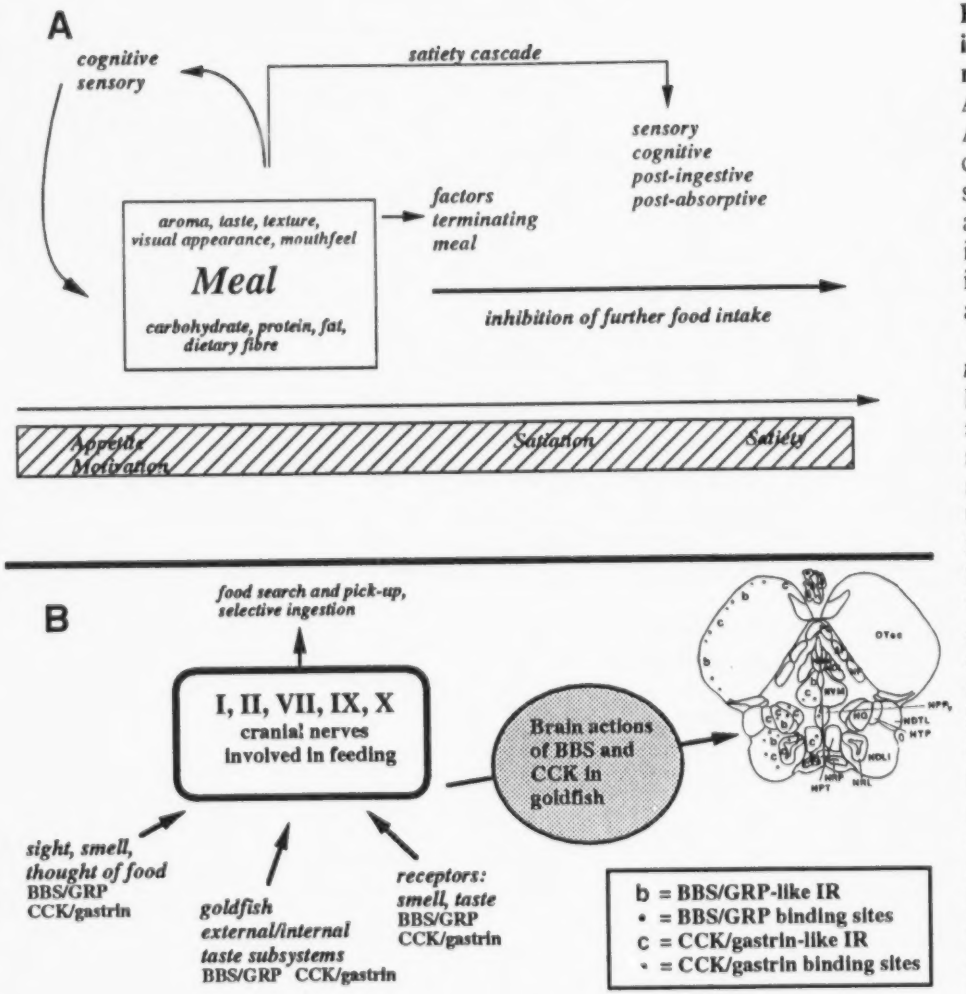

Fig. 9-1. Concept of feeding behavior and involvement of BBS and CCK in the regulation of food intake in goldfish.
A) ***Concept of feeding behavior in goldfish.*** Appetite motivation and meal consumption are dependent on sensory information from the food source including the aroma, taste, texture, visual appearance, and mouthfeel. Intake of a meal initiates a satiety cascade which is based on the integration of sensory, cognitive, post-ingestive and post-absorptive factors.
B) ***Involvement of CCK and BBS in the regulation of food intake in goldfish.*** The hypothalamic feeding center of the goldfish brain serves as an integrating center for incoming sensory stimuli with respect to the food source and feeding. The palatability and selectivity of the food to be consumed is dependent on sensory information which is received via the vagal and facial nerves, and subsequently transmitted to the vagal and facial lobes of the hindbrain. Visual and aroma sensations are detected via the olfactory and optic nerves, and information is passed to the telencephalon. All sensory information is then transmitted to the goldfish hypothalamic inferior lobes, where the integration of sensory information results in modified feeding behavior. BBS and CCK appear to be involved in both the reception of sensory feeding information outside of the hypothalamic feeding area, as well as in the local release and integration of incoming feeding stimuli within the brain feeding center to alter food intake in the goldfish.

mammals, it has been reported that elevations in GH precede feeding activity, and that GH may actually facilitate feeding behavior (Even *et al.*, 1987). It was therefore speculated that a similar event may occur in the goldfish with respect to GH-induced feeding, and that BBS had "clamped" serum GH levels thereby reducing food intake.

However, further studies suggested that this initial clamping of GH levels in BBS - injected goldfish was probably the result of unchanged serum GH levels representative of an "unfed" fish (since BBS administration results in decreased food intake levels). On the contrary, elevations in serum GH, which were present in BBS-injected fish at 45 minutes, were probably the result of direct actions of BBS at the level of the goldfish pituitary. The lack of an acute effect of altered serum GH levels on regulating feeding activity in goldfish is consistent with preliminary studies which demonstrated that ip injection of carp growth hormone releasing factor (cGRF) into goldfish resulted in no change in cumulative food intake within 45 minutes, but produced an elevation in serum GH levels. Furthermore, inconsistent changes in circulating levels of serum GH in goldfish which had been icv or ip -injected with CCK8-s despite suppressed feeding effects, indicated that alterations in serum GH levels are likely a consequence of modified feeding activity or peptide injection. Goldfish which received either BBS of CCK8-s exhibited no alterations in circulating levels of serum GtH-II. Likewise, experiments in Chapter 2 also demonstrated little change in serum GtH-II levels following feeding of varied rations to fish, indicating that GtH-II does not participate in the acute regulation of feeding behavior in goldfish.

Concomitant changes in circulating levels of GH following CCK8-s and BBS administration into goldfish which do not appear to be feeding-related, suggest that CCK/gastrin-like peptides and BBS/GRP-like peptides may act within the goldfish to regulate anterior pituitary hormone secretion. Several findings in this thesis confirmed this hypothesis, by providing evidence for a direct action of CCK/gastrin-like peptides and BBS/GRP-like peptides at the level of the goldfish pituitary. Firstly, the presence of both CCK/gastrin-like IR and BBS/GRP-like IR material was demonstrated in the goldfish anterior pituitary. In particular, CCK/gastrin-like IR nerve fibres were localized within the proximal PD of the anterior pituitary, and were found to be co-distributed amongst both the somatotropes and gonadotropes. Secondly, preliminary studies in

Chapter 7 and Chapter 8 have detected ^{125}I-BH-CCK8-s and ^{125}I-[Tyr-4]-BBS-14 binding sites in the anterior goldfish pituitary, suggesting that CCK/gastrin-like peptides and BBS/GRP-like peptides have direct actions at the level of the pars distalis. Third, when goldfish pituitary fragments were challenged with repeated pulses of low doses of either CCK8-s (0.1, 1.0 or 10 nM) or BBS (0.1, 1.0 or 10 nM), an increase in the secretion of both GH and GtH-II resulted. Likewise, perifusion of pituitary fragments with several CCK/gastrin and BBS/GRP -related peptides also resulted in the release of GH and GtH-II, indicating that members of the CCK/gastrin family and BBS/GRP family of peptides likely act as physiological regulators of anterior pituitary hormone release.

Finally, immunohistochemical studies presented within this thesis provide anatomical evidence that CCK/gastrin-like and BBS/GRP-like peptides, which regulate the release of GH and GtH-II from the pituitary, likely originate in the hypothalamus. Studies have indicated that CCK/gastrin-like IR and BBS/GRP-like IR material is distributed within the nucleus preopticus (NPO), the nucleus preopticus periventricularis (NPP), and the nucleus lateralis tuberis (NLT) of the hypothalamus, as well as within the hypophysial stalk. These brain regions all send neuronal projections to the anterior pituitary, which are reported to be involved in the regulation of anterior GH and GtH-II release in the goldfish (Peter and Fryer, 1983). Based on these findings it is suggested that in the goldfish, CCK/gastrin-like peptides and BBS/GRP-like peptides are produced within neurons of specific hypothalamic areas; these neurons then project their terminals into the anterior pituitary where CCK/gastrin and BBS/GRP -like peptides regulate the release of GH and GtH-II from the somatotropes and gonadotropes.

How is the release of CCK/gastrin and BBS/GRP originating from the hypothalamus regulated? Since preliminary data in Chapters 7 and 8 indicate the presence of binding sites for ^{125}I-BH-CCK8-s and ^{125}I-[Tyr-4]-BBS-14 in the NPO, NPP, and the NLT, which are areas that are partially located outside of the blood brain barrier (Peter *et al.*, 1980), it is possible that circulating CCK/gastrin and/or BBS/GRP can autoregulate hypothalamic CCK/gastrin and/or BBS/GRP release following binding at these designated brain sites. Alternatively, the secretion of hypothalamic CCK/gastrin-like and BBS/GRP-like peptides may also be regulated through the interactions with other known hypothalamic regulators of GH and GtH-II release, such as salmon gonadotropin

releasing hormone (sGnRH) (Marchant *et al.*, 1989), or somatostatin (Marchant *et al.*, 1989).

Presently, it is unknown why CCK/gastrin-like and BBS/GRP-like peptides function to regulate GH and GtH-II release in the goldfish. In fish, it is known that energy is continually shunted between phases of body growth and reproduction. It is plausible that CCK/gastrin-like and BBS/GRP-like peptides participate in regulating GH release, and the effects GH have on long-term feeding strategies and body growth. Additionally, CCK/gastrin-like and BBS/GRP-like peptides may play a fundamental role in regulating the release of GtH-II during spawning. Concurrently, during the reproductive phase in teleosts, CCK/gastrin-like and BBS/GRP-like peptides may act to decrease feeding behavior and augment sexual behavior.

Findings in this thesis of a role for both CCK/gastrin and BBS/GRP in the regulation of feeding behavior and anterior pituitary hormone secretion in the goldfish demonstrate that these peptides have widespread roles within the teleost central nervous system, and provide a foundation from which future research can continue into this novel area.

9.4 References

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Appendix 1

Nutritional Composition of Diets #1 and #2

Table A1-1. Nutritional composition of diet #1
(floating pellets EXSL 440; extruded salmon diet).

Crude protein, minimum	43%
Crude fat, minimum	17%
Crude fibre, minimum	5%
Ash, maximum	12%
Sodium, actual	0.5%
Phosphorus, actual	1.3%
Calcium, actual	1.8%
Vitamin A, minimum	8000 iu/kg
Vitamin D, minimum	2400 iu/kg
Vitamin E, minimum	150 iu/kg

Rangen, Inc., Buhl, Idaho, pellet size 5/32.

Table A1-2. Nutritional composition of diet #2
(floating trout pellets)

Crude protein, minimum	40%
Crude fat, minimum	11%
Crude fibre, minimum	3%
Sodium, actual	0.35%
Phosphorus, actual	0.9%
Calcium, actual	1.1%
Vitamin A, minimum	7500 iu/kg
Vitamin D, minimum	3000 iu/kg
Vitamin E, minimum	100iu/kg

Martin Feed Mills Limited, Elmira, pellet size 1/4.

Appendix 2

Preliminary Studies on the In Vitro Effects of Selected BBS Antagonists on Anterior Pituitary Hormone Release in Goldfish

A2.1 Introduction

In the goldfish, the presence of BBS/GRP-like IR material (Himick and Peter, 1994b) and specific, high affinity BBS/GRP receptor binding sites within the brain and pituitary (Chapter 7), indicate that members of BBS/GRP-like peptides are involved in fundamental physiological processes within the teleost central nervous system. In support of this, it has recently been demonstrated that BBS/GRP-like peptides play a role in the central regulation of feeding behavior in goldfish (Himick and Peter, 1994a). Furthermore, BBS/GRP-like peptides act to release both growth hormone (GH) and gonadotropin (GtH-II) from goldfish pituitary fragments *in vitro* (Himick and Peter, 1994b). While actions mediated by BBS/GRP-like peptides on feeding behavior and anterior pituitary hormone release in goldfish appear specific with respect to the peptide structural sequence and/or the dose administered, the effects of selected BBS/GRP-like antagonists on such actions would further confirm the specificity of BBS/GRP-like peptide actions in the control of food intake and GH and GtH-II secretion in goldfish. To our knowledge, the existence of an adequate BBS-antagonist has not been reported for fish or any other lower vertebrate (S. Vigna, Duke University, Durham, NC; pers. comm.).

In mammals, several classes of BBS antagonists have been synthesized and their effects have been examined both *in vitro* and *in vivo*. Substance P derivatives and BBS derivatives have low potency with variable specificity. BBS derivatives with a reduced peptide bond and GRP derivatives containing an amino-terminal ester and a C-terminal amide or alkyl ether have also been synthesized, but have been reported to act both antagonistically and as an agonist. For example, one of the most potent BBS antagonist analogues [Leu^{13},$\Psi(CH_2NH)$ Leu^{14}]-BBS is successful in inhibiting BBS-stimulated pancreatic amylase secretion *in vivo* (Alptekin *et al.*, 1991), but fails to inhibit GH release *in vivo* following brain injection (Houben and Denef, 1990) and may be partially

agonistic on prolactin release when examined *in vitro* following incubation with pituitary cell aggregates (Houben and Denef, 1991). Thus, even in mammalian systems BBS antagonists have been shown to lack specificity in their actions.

In this chapter, preliminary studies have investigated the effects of several selected BBS-antagonists on the release of anterior pituitary hormone secretion from goldfish pituitary fragments *in vitro*. These studies represent an initial investigation into the discovery of a suitable BBS/GRP-like antagonist which will act selectively at the level of the goldfish BBS/GRP receptor binding site, thus permitting studies on the specificity of BBS-mediated actions of feeding behavior in fish.

A2.2 Materials and Methods

Experimental Animals

Male and female goldfish (25-45 g; Ozark Fisheries, Stoutland, MO) were maintained under simulated Edmonton natural photoperiod in 1.8 kL tanks which received a constant flow of aerated and dechlorinated city water at 17^{O}C. Fish were fed *ad libitum* twice daily with commercially prepared fish pellets until used for *in vitro* perifusion experiments.

Perifusion of Goldfish Pituitary Fragments with BBS Antagonists

The effects of BBS/GRP-like antagonists were examined using the goldfish pituitary fragment perifusion system described previously in Chapter 4. Fish were anesthetized, killed, and pituitaries were quickly removed and cut into fragments. Fragments (equivalent to 3 pituitaries/column) were perifused overnight with medium 199 containing Hank's Balanced Salts, followed by a 2 h pre-perifusion of Hank's Balanced Salt Solution. In all experiments, the perifusion flow rate was 15 mL/h, and 1.25 mL fractions were collected using an automatic fraction collector. The samples were stored at -25^{O}C until determination of GH and GtH-II levels by use of specific RIAs developed for common carp GH and GtH-II, as outlined in Chapter 2.

Data Analyses and Statistics

For *in vitro* perifusion experiments, the GH release responses to each pulse of BBS or BBS-antagonist were determined by quantifying the net response above average basal levels. Basal level was defined as the average hormone content in the three fractions collected immediately preceding each pulse (prepulse). Net responses were expressed as a percentage of the prepulse value. A release response was considered to be terminated when hormone content was within one standard error (SEM) of the mean prepulse value. The transformation to percentage of prepulse hormone levels allowed results from several columns to be combined for statistical analysis. Differences in the hormone responses to pulses of BBS were compared by one-way analysis of variance (ANOVA) followed by Student-Newmann Keuls multiple comparison test. For all experiments, significance was at $p \leq 0.05$.

Drugs Administered

BBS-14 was purchased from Bachem Bioscience Inc. (Philadelphia, PA). The BBS antagonists [D-Phe^{12}]-BBS, [D-Phe^{12},Leu^{14}]-BBS, and [Leu13-ψ(CH_2NH)Leu^{14}]-BBS were purchased from Sigma. Synthetic sGnRH was purchased from Penninsula Laboratories (Belmont, CA) and was diluted with HHBSA from stock solution (10 µg/20 µL in 0.1 N acetic acid) immediately prior to use in *in vitro* perifusion experiments.

A2.3 Experiments and Results

Effects of selected BBS antagonists on anterior pituitary hormone release.

Fig. A2-1 and Fig. A2-2 represent the mean GH and GtH-II (Fig. A2-1) release responses from pituitary fragments of regressed goldfish which were exposed to repeated five minute pulses of either 100 nM BBS or one of the several selected BBS antagonists, [D-Phe^{12}] BBS (10 µM), [D-Phe^{12}, Leu^{14}] BBS (10 µM) (Fig. A2-1) or [Leu^{13}-ψ(CH_2NH) Leu^{14}] BBS (1 µM) (Fig. A2-2). BBS antagonists were administered for 30 minutes, followed by a single five minute pulse of 100 nM BBS in the presence of BBS antagonist. Between testing the effects of BBS antagonist actions on pituitary hormone release within an experiment, fragments were perifused with media for 45 or 50 minutes (washing stage).

When calculated as the mean total hormone release per pulse (expressed as a

percentage of basal prepulse levels), repeated challenge of 100 nM BBS resulted in a significant elevation in GH (64%, 59% and 23% net release over basal for pulses 1, 2, and 3, respectively) and GtH-II (122%, 50%, 109% over basal for pulses 1, 2, and 3, respectively) secretion (Fig. A2-1). On the contrary, challenge of 100 nM BBS in the presence of [D-Phe^{12}] BBS resulted in a net total GH and GtH-II release response of 706% and 828% over basal, respectively. In the presence of [D-Phe^{12}, Leu^{14}] BBS, 100 nM BBS produced a 20% and 76% net increase in total GH and GtH-II release, respectively. Finally, a third pulse of 100 nM BBS following perifusion of the two BBS antagonists resulted in a 14% and 11% increase in net total GH and GtH-II release, respectively. In all columns, a three minute control pulse of sGnRH at the end of the experiment stimulated both GtH-II and GH release.

In Fig. A2-2, two repeated five minute pulses of 100 nM BBS produced a 23% and 31% increase in total amount of GH release over basal, while challenge of 100 nM BBS in the presence of the BBS antagonist [Leu^{13} ψ (CH_2NH) Leu^{14}] BBS produced an inhibition in the total amount of GH released to -55% below basal levels. A second pulse of 100 nM BBS which followed BBS antagonist challenge, resulted in a 36% increase in the total amount of GH released above basal. In all columns, a five minute control pulse of sGnRH at the end of the experiment stimulated GH release.

A2.4 Discussion

In mammals, it has been shown that four different classes of BBS receptor antagonists exist (for review Von Schrenck *et al.*, 1990). The first class includes the D-amino-substituted substance P analogues that function as SP receptor antagonists, but also have been shown to act as weak BBS receptor antagonists. The second class includes the D-amino acid substituted analogues of BBS, while the third class includes the reduced peptide bond analogues to which [Leu^{13} ψ (CH_2NH) Leu^{14}] BBS belongs. The fourth class of BBS receptor antagonists includes des-Met analogues of the COOH-terminus of GRP or BBS. While the actions of selected BBS antagonists from these four classes have been examined in mammals both *in vivo* and *in vitro*, their possible antagonistic actions of BBS in lower vertebrates remain unknown.

In the present study, preliminary findings indicate that the BBS antagonist [Leu^{13} ψ

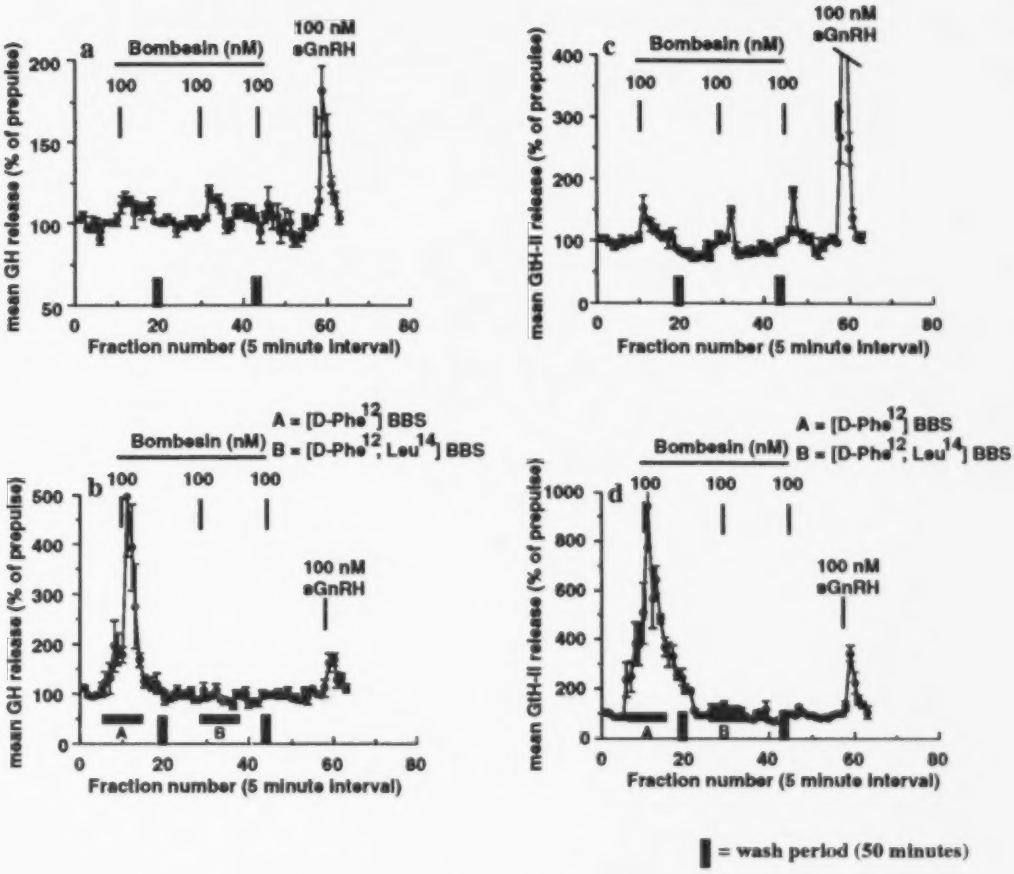
(CH_2NH) Leu^{14}] BBS was effective in antagonizing BBS-mediated actions on anterior pituitary hormone release from goldfish pituitary fragments *in vitro*. When fragments were perifused for 30 minutes with [Leu^{13} ψ (CH_2NH) Leu^{14}] BBS, no change in basal levels occurred until challenge with 100 nM BBS (Fig. A2-2). During this pulse, the GH release response to BBS was inhibited, indicating that in the presence of [Leu^{13} ψ (CH_2NH) Leu^{14}] BBS, BBS actions are blocked. Such findings support studies in perifused rat anterior pituitary cell aggregates, where [Leu^{13} ψ (CH_2NH) Leu^{14}] BBS has been shown to inhibit the GH release response to the GRP peptide, neuromedin C (Houben and Denef, 1991). Recently, [Leu^{13} ψ (CH_2NH) Leu^{14}] BBS has also been reported to act within the brain, by blocking the suppressive effects of centrally administered BBS on food intake in satiated rats (Merali *et al.*, 1993).

On the contrary, these studies also revealed that the BBS analogue [D-Phe^{12}] BBS may act independently as an agonist of goldfish pituitary hormone secretion, based on the release responses obtained with GH and GtH-II prior to BBS challenge (Fig. A2-1). In the presence of [D-Phe^{12}] BBS, BBS challenge resulted in a considerably higher increase in the total amount of GH and GtH-II release relative to the hormone release responses obtained in parallel controls (fragments which were challenged with BBS only, and no BBS antagonist). The effects of [D-Phe^{12}] BBS at the level of the goldfish pituitary appear to be stimulatory, unlike the mammalian system where this same BBS analogue functions as a BBS receptor antagonist centrally, as well as peripherally at the level of the pancreas (Heinz-Erian *et al.*, 1987; Merali *et al.*, 1988).

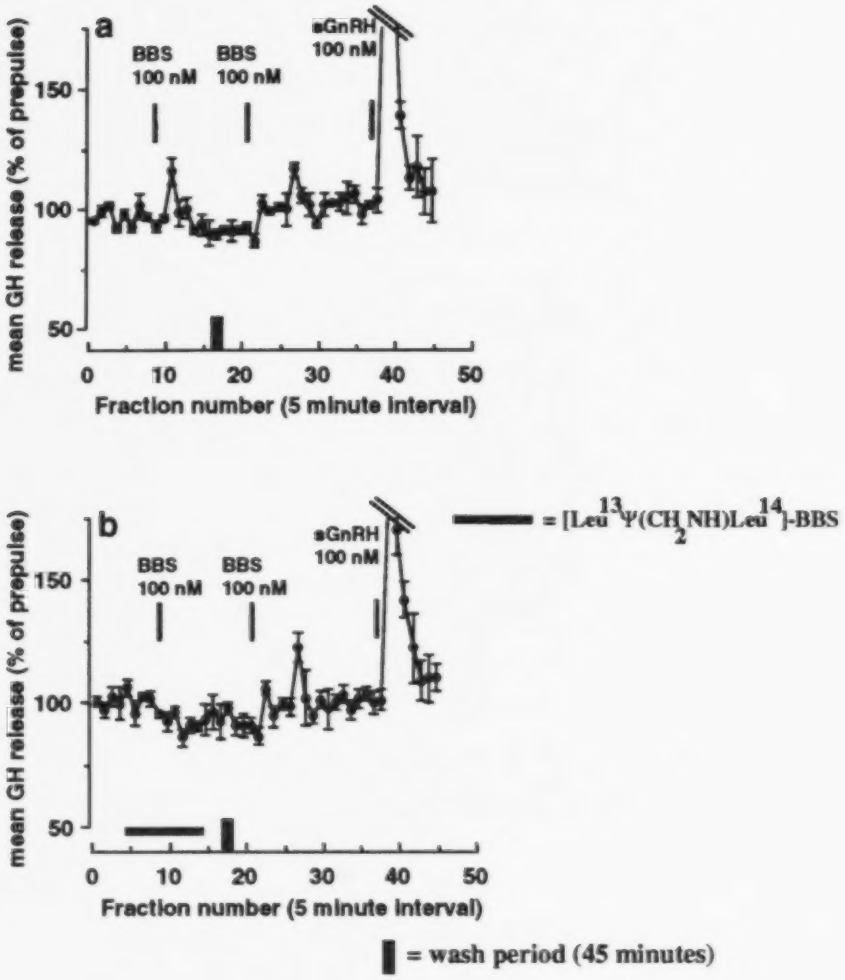
Finally, the presence of the D-amino acid substituted BBS analogue [D-Phe^{12}, Leu^{14}] BBS did not independently alter basal GH and GtH-II levels prior to BBS challenge. Following a pulse of BBS, the mean total GH and GtH-II released was comparable to control release responses, where only BBS was administered in the absence of BBS antagonist. [D-Phe^{12}, Leu^{14}] does not appear to function as an antagonist at the level of the goldfish central BBS/GRP receptor binding site.

Such preliminary findings presented herein suggest that [Leu^{13} ψ (CH_2NH) Leu^{14}] BBS may act as a selective antagonist within the goldfish central nervous system at the level of the BBS/GRP-like receptor. Studies to examine the binding affinity and specificity of [Leu^{13} ψ (CH_2NH) Leu^{14}] BBS at the goldfish brain BBS/GRP binding

site, as well as additional *in vitro* investigation of this analogue using the goldfish pituitary fragment system, will provide valuable information with respect to the use of [Leu^{13} ψ (CH_2NH) Leu^{14}] BBS in examining the specificity of BBS-mediated suppression of feeding behavior in the goldfish.



A2-1. Release profiles of GH and GtH-II in perifusion columns following challenge with BBS antagonists. Pituitary fragments from gonadal regressed goldfish were exposed to three five minute pulses of 100 nM BBS (**a** and **c**; controls) or the BBS antagonists [D-Phe 12] BBS or [D-Phe 12, Leu 14] BBS (**b** and **d**) (10 uM; horizontal bar represents perifusion of BBS antagonist). At 30 minutes following BBS antagonist challenge, a single five minute pulse of BBS (100 nM) was administered in the presence of antagonist. Perifusion of BBS antagonist continued for 10 minutes following BBS challenge. At the end of the experiment, a 3 minute pulse of 100 nM sGnRH was administered as a control. n = 4 columns/experiment; mean hormone basal levels prior to peptide challenge, GH = 11.5 ng/mL; GtH-II = 7.8 ng/mL.



A2-2. Release profiles of GH in perifusion columns following challenge with BBS antagonist. Pituitary fragments from gonadal regressing goldfish were exposed to two five minute pulses of 100 nM BBS (**a**; control) or the BBS antagonist [Leu13Ψ(CH2NH)Leu14]-BBS (**b**) (1.0 uM; horizontal bar represents perifusion of BBS antagonist). At 30 minutes following BBS antagonist challenge, a single five minute pulse of BBS (100 nM) was administered in the presence of antagonist. Perifusion of BBS antagonist continued for 15 minutes following BBS challenge. At the end of the experiment, a 5 minute pulse of 100 nM sGnRH was administered as a control. n = 4 columns/experiment; mean hormone basal levels prior to peptide challenge, GH = 13.0 ng/mL.

A2.5 References

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